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Date: 16 December 1999 By WINSOME SINCLAIR

METHODS AND COMPOSITIONS FOR SCREENING FOR ANGIOGENESIS MODULATING COMPOUNDS

TECHNICAL FIELD

This invention is in the field of molecular biology and medicine. More specifically, it relates to novel vector constructs, compositions, and methods of use thereof for screening compounds in host cells or transgenic animals. Further, the invention relates to vector constructs and methods of use thereof to generate transgenic organisms, particularly transgenic mice.

BACKGROUND

A requirement for cellular inflow of nutrients, outflow of waste products, and gas exchange in most tissues and organs is the establishment of a vascular supply. Several processes for blood vessel development and differentiation have been identified. One such process is termed "vasculogenesis" and takes place in the embryo. This process consists of the *in situ* differentiation of mesenchymal cells into hemoangioblasts, which are the precursors of both endothelial cells and blood cells. "Angiogenesis" is a second such process and involves the formation of new blood vessels from a preexisting endothelium. This process is required for (i) the development of embryonic vasculature, and (ii) a variety of post-natal processes, including, but not limited to, wound healing, tissue regeneration, and organ regeneration. Further, angiogenesis has been identified as a requirement for solid tumor growth and uncontrolled blood cell proliferation.

Vascular Endothelial Growth Factor (VEGF; also designated as vascular permeability factor (VPF) has been identified as a regulator of normal and pathological

angiogenesis. VEGF is a secreted growth factor having the following properties: (i) an endothelial cell specific mitogen; (ii) angiogenic *in vivo* and induces vascular permeability; (iii) VEGF expression (and expression of its receptors) has been correlated with vasculogenesis and angiogenesis during embryonic development; and (iv) VEGF is expressed in tumor cells. The VEGF receptor appears to be expressed exclusively in adjacent small blood vessels. VEGF appears to play a crucial role in the vascularization of a wide range of tumors including, but not limited to, breast cancers, ovarian tumors, brain tumors, kidney and bladder carcinomas, adenocarcinomas and malignant gliomas. Tumors have been shown to produce ample amounts of VEGF which stimulates the proliferation and migration of endothelial cells (ECs). This is thought to induce tumor vascularization by a paracrine mechanism.

The angiogenic effect of VEGF appears to be mediated by its binding to high affinity cell surface VEGF receptors (VEGFR).

SUMMARY OF THE INVENTION

The invention provides methods and compositions relating to the VEGFR-2 gene transcriptional promoter. The compositions include recombinant regulators of gene expression comprising the VEGFR-2 promoter of SEQ ID NO:32, further sequences isolated based on the teachings disclosed herein, or deletion mutants thereof, typically at least 10, 20, 25, 50, or 100 bp in length, where the sequences maintain cis transcriptional regulatory activity.

The invention also provides hybridization probes and replication/amplification primers having a hitherto novel VEGFR-2 specific sequence contained in SEQ ID NO:32 (including its complement and analogs and complements thereof having the corresponding sequence, e.g., in RNA) and sufficient to effect specific hybridization thereto (i.e., specifically hybridize with SEQ ID NO:32 in the presence of genomic DNA).

The invention also provides cells and vectors comprising the disclosed VEGFR-2 regulators, including cells comprising such regulators operably linked to

non-VEGFR-2 coding sequences (i.e., a heterologous coding sequence). Such cells find use in the disclosed methods for identifying agents or compounds that regulate the activity of a VEGFR-2 promoter. In an exemplary method, the cells are contacted with a candidate agent, under conditions wherein, but for the presence of said agent, the VEGFR-2 promoter exhibits a first expression of a reporter; detecting the presence of a second expression of the reporter, wherein a difference between said first and said second expression of the reporter indicates that the agent affects the expression mediated by the VEGFR-2 promoter.

In another aspect, this invention relates to a substantially purified nucleic acid molecule comprising a VEGFR-2 promoter region (i.e., an isolated polynucleotide). In one embodiment the isolated polynucleotide comprises the sequence presented as SEQ ID NO:32, and fragments thereof which maintain cis-acting transcriptional activity, in particular, a regulator of gene expression derived from SEQ ID NO:32 wherein said polynucleotide sequence has cis transcriptional regulatory activity. A further embodiment includes, an isolated polynucleotide comprising, a cis-acting transcription regulator having X contiguous nucleotides, wherein (i) the X contiguous nucleotides have at least about 90% identity to Y contiguous nucleotides derived from SEQ ID NO:32, (ii) X equals Y, and (iii) X is greater than or equal to 50. Exemplary values of X include, but are not limited to, X is greater than or equal to 500, X is greater than or equal to 3563, X is in the range of 50-3570 including all integer values in that range.

The invention also includes an expression cassette comprising the above-described polynucleotides. The invention also relates to expression vectors comprising the aforementioned polynucleotide sequences and host cells transformed with these expression vectors.

In another aspect, the invention also relates to methods for detecting test agents which modulate transcription of the VEGFR-2 promoters described above. Such methods include contacting a host cell transformed with an expression vector comprising the VEGFR-2 promoter DNA sequence operably linked to a reporter sequence with the test agent and comparing the level of transcription produced by the test agent to the level of transcription produced in its absence.

The invention also relates to transgenic or chimeric animals whose cells express a heterologous gene under the transcriptional control of a VEGFR-2 promoter, and methods of using such animals as described herein.

In another aspect, the invention relates to the above embodiments wherein the promoter sequence is derived from Tie2.

These and other embodiments of the present invention will be apparent to those of skill in the art in view of the teachings herein.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic depicting construction of the pTK53 vector. Polynucleotides encoding PGK-P, Neo and TK and 5' and 3' linkers are introduced into a pKS backbone to produce the vector designated pTK53.

Figure 2 is schematic depicting construction of the pTK-LucR and pTK-LucYG vectors. For pTK-LucR, a polynucleotide encoding LucR is introduced into pTK53. Thus, the pTK-LucR construct contains the PGK-P gene, a neomycin (Neo^r) gene, a thymidine kinase (TK) gene and sequence encoding red luciferase (Luc-R). For pTK-LucYG, a polynucleotide encoding LucYG is introduced into pTK53. Thus, the pTK-LucYG construct contains the PGK-P gene, a neomycin (Neo^r) gene, a thymidine kinase (TK) gene and a sequence encoding yellow-green luciferase (Luc-YG).

Figures 3A is a schematic depicting the vector pTKLR-Vn. Sequences homologous to the vitronectin gene are inserted into pTK-LucR such that they flank the Neo^r gene and the Luc-R coding sequence. Figure 3B is a schematic depicting targeting of the linearized pTKLR-Vn vector to the vintronectin chromosomal locus. The VEGF promoter is cloned into the polylinkers between Neo and Luc-R. Upon homologous recombination, the Neo-VEGF-LucR transgene is inserted into the Vn gene. In the figure, (A) shows the targeting vector pTKLR-Vn and (B) shows the mouse vitronectin gene. In the figure, Neo – neomycin resistance encoding sequences; TK – thymidine kinase encoding sequences; LucR – red luciferase from pGL3Red (Dr. Christopher Contag, Stanford University, Stanford, CA). Regions bearing Vn gene translational start and stop codons are indicated with arrows.

Poly(A) sequences are placed upstream of the polylinker to prevent or minimize read-through translation. Figure 3C shows the nucleotide sequence of vitronectin.

Figure 4A is a schematic depicting the vector pTKLG-Fos. Sequences homologous to the FosB gene are inserted into pTK-LucYG such that they flank the Neo^r gene and the Luc-YG coding sequence. Figure 4B shows the nucleotide sequence of FosB.

Figures 5A, 5B, and 5C depicts the nucleotide sequence of the entire promoter region of the VEGFR2 mouse gene (SEQ ID NO:32).

Figure 6 depicts PCR conditions for genomic screening for promoters useful in exemplary targeting constructs of the present invention.

Figure 7 depicts generation of targeted transgenic mice using the targeting vectors described herein.

Figure 8 depicts of schematic representation of Southern blot analysis of homologous DNA recombination between pTKLG-Fos targetting vector and the FosB gene.

Figure 9 depicts generation of targeted transgenic mice, using the targeting vectors described herein, and crosses using such transgenics as well as their offspring (F1, first generation; F2, second generation).

Figure 10 depicts crosses using transgenic mice of the present invention to generate dual luciferase transgenic mice.

Figure 11 depicts the nucleotide sequence of a 511 bp enhancer region of VEGFR2 (SEQ ID NO:35).

Figure 12 is a schematic depicting engineering of the pGL3B2-KPN construct. PGL3B (Promerga, Madison, WI) contains the yellow-green luciferase gene (Luc-YG). The construct contains a 4.5 kb fragment of the VEGFR2 promoter and a 0.5 kb fragment of the VEGFR2 enhancer.

Figure 13 is a schematic depicting engineering of the pTKLG-Fos-KPN construct made using pGL3B2-KPN (Figure 12) and pTKLG-Fos.

Figure 14 is a schematic depicting targeting of the linearized pTKLG-Fos vector to the FosB chromosomal locus. The VEGFR2 promoter is cloned into the polylinkers between Neo and Luc-YG. Upon homologous recombination, the Neo-VEGFR2-LucYG

transgene will be inserted into a sequence associated with production of FosB. In the figure, (A) shows the targeting vector, and (B) shows the mouse target gene. In the figure, Neo – neomycin resistance encoding sequences; TK – thymidine kinase encoding sequences; LucYG – yellow green luciferase from pGL3-control vector (Promega, Madison, WI). Regions bearing FosB gene translational start and stop codons are indicated with arrows. Poly(A) sequences are placed upstream of the polylinker to prevent or minimize read-through translation.

Figure 15 depicts the nucleotide sequence of the entire promoter region of the Tie2 mouse gene (SEQ ID NO:40).

Figure 16 depicts the nucleotide sequence of a 1.7 kb enhancer region of Tie2 (SEQ ID NO:41).

Figure 17 is a schematic depicting engineering of the pGL3B2-TPN construct. PGL3B (Promerga, Madison, WI) contains the yellow-green luciferase gene (Luc-YG). The construct contains a 7.1 kb fragment of the Tie2 promoter and a 1.7 kb fragment of the Tie2 enhancer.

Figure 18 is a schematic depicting engineering of the pTKLG-Fos-KPN construct made using pGL3B2-KPN (Figure 17) and pTKLG-Fos.

Figure 19 is a schematic depicting targeting of the linearized pTKLG-Fos vector to the FosB chromosomal locus. The TIE2 promoter is cloned into the polylinkers between Neo and Luc-R. Upon homologous recombination, the Neo-Tie2-LucYG transgene is inserted into the FosB gene. In the figure, (A) shows the targeting vector, and (B) shows the mouse target gene. In the figure, Neo – neomycin resistance encoding sequences; TK – thymidine kinase encoding sequences; LucYG – yellow green luciferase from pGL3-control vector (Promega). Regions bearing FosB gene translational start and stop codons are indicated with arrows. Poly(A) sequences are placed upstream of the polylinker to prevent or minimize read-through translation.

MODES FOR CARRYING OUT THE INVENTION

Throughout this application, various publications, patents, and published patent applications are referred to by an identifying citation. The disclosures of these publications, patents, and published patent specifications referenced in this application are hereby incorporated by reference into the present disclosure to more fully describe the state of the art to which this invention pertains.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, cell biology and recombinant DNA, which are within the skill of the art. *See, e.g.,* Sambrook, Fritsch, and Maniatis, MOLECULAR CLONING: A LABORATORY MANUAL, 2nd edition (1989); CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, (F.M. Ausubel et al. eds., 1987); the series METHODS IN ENZYMOLOGY (Academic Press, Inc.); PCR 2: A PRACTICAL APPROACH (M.J. McPherson, B.D. Hames and G.R. Taylor eds., 1995) and ANIMAL CELL CULTURE (R.I. Freshney. Ed., 1987).

All publications, patents and patent applications cited herein, whether *supra* or *infra*, are hereby incorporated by reference in their entirety.

As used in this specification and the appended claims, the singular forms "a," "an" and "the" include plural references unless the content clearly dictates otherwise. Thus, for example, reference to “an antigen” includes a mixture of two or more such agents.

Definitions

As used herein, certain terms will have specific meanings.

The terms "nucleic acid molecule" and "polynucleotide" are used interchangeably to and refer to a polymeric form of nucleotides of any length, either deoxyribonucleotides or ribonucleotides, or analogs thereof. Polynucleotides may have any three-dimensional structure, and may perform any function, known or unknown. Non-limiting examples of polynucleotides include a gene, a gene fragment, exons, introns, messenger RNA (mRNA), transfer RNA, ribosomal RNA, ribozymes, cDNA, recombinant polynucleotides, branched

polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers.

A polynucleotide is typically composed of a specific sequence of four nucleotide bases: adenine (A); cytosine (C); guanine (G); and thymine (T) (uracil (U) for thymine (T) when the polynucleotide is RNA). Thus, the term polynucleotide sequence is the alphabetical representation of a polynucleotide molecule. This alphabetical representation can be input into databases in a computer having a central processing unit and used for bioinformatics applications such as functional genomics and homology searching.

A "coding sequence" or a sequence which "encodes" a selected polypeptide, is a nucleic acid molecule which is transcribed (in the case of DNA) and translated (in the case of mRNA) into a polypeptide, for example, *in vivo* when placed under the control of appropriate regulatory sequences (or "control elements"). The boundaries of the coding sequence are typically determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxy) terminus. A coding sequence can include, but is not limited to, cDNA from viral, procaryotic or eucaryotic mRNA, genomic DNA sequences from viral or procaryotic DNA, and even synthetic DNA sequences. A transcription termination sequence may be located 3' to the coding sequence. Other "control elements" may also be associated with a coding sequence. A DNA sequence encoding a polypeptide can be optimized for expression in a selected cell by using the codons preferred by the selected cell to represent the DNA copy of the desired polypeptide coding sequence. "Encoded by" refers to a nucleic acid sequence which codes for a polypeptide sequence, wherein the polypeptide sequence or a portion thereof contains an amino acid sequence of at least 3 to 5 amino acids, more preferably at least 8 to 10 amino acids, and even more preferably at least 15 to 20 amino acids from a polypeptide encoded by the nucleic acid sequence. Also encompassed are polypeptide sequences which are immunologically identifiable with a polypeptide encoded by the sequence.

A "transcription factor" as used herein typically refers to a protein (or polypeptide) which affects the transcription, and accordingly the expression, of a specified gene. A

transcription factor may refer to a single polypeptide transcription factor, one or more polypeptides acting sequentially or in concert, or a complex of polypeptides.

Typical "control elements", include, but are not limited to, transcription promoters, transcription enhancer elements, transcription regulating elements (transcription regulators), transcription termination signals, polyadenylation sequences (located 3' to the translation stop codon), sequences for optimization of initiation of translation (located 5' to the coding sequence), translation enhancing sequences, and translation termination sequences. Transcription promoters can include inducible promoters (where expression of a polynucleotide sequence operably linked to the promoter is induced by an analyte, cofactor, regulatory protein, etc.), repressible promoters (where expression of a polynucleotide sequence operably linked to the promoter is induced by an analyte, cofactor, regulatory protein, etc.), and constitutive promoters. A transcription regulator is a cis-acting element that affects the transcription of a gene, for example, a region of a promoter with which a transcription factor interacts to induce expression of a gene.

"Expression enhancing sequences" typically refer to control elements that improve transcription or translation of a polynucleotide relative to the expression level in the absence of such control elements (for example, promoters, promoter enhancers, enhancer elements, and translational enhancers (e.g., Shine and Delagarno sequences)).

"Purified polynucleotide" refers to a polynucleotide of interest or fragment thereof which is essentially free, e.g., contains less than about 50%, preferably less than about 70%, and more preferably less than about 90%, of the protein with which the polynucleotide is naturally associated. Techniques for purifying polynucleotides of interest are well-known in the art and include, for example, disruption of the cell containing the polynucleotide with a chaotropic agent and separation of the polynucleotide(s) and proteins by ion-exchange chromatography, affinity chromatography and sedimentation according to density.

A "heterologous sequence" as used herein is typically refers to either (i) a nucleic acid sequence that is not normally found in the cell or organism of interest, or (ii) a nucleic acid sequence introduced at a genomic site wherein the nucleic acid sequence does not normally occur in nature at that site. For example, a DNA sequence encoding a polypetide

can be obtained from yeast and introduced into a bacterial cell. In this case the yeast DNA sequence is "heterologous" to the native DNA of the bacterial cell. Alternatively, a promoter sequence from a Tie2 gene can be introduced into the genomic location of a *fosB* gene. In this case the Tie2 promoter sequence is "heterologous" to the native *fosB* genomic sequence.

A "polypeptide" is used in it broadest sense to refer to a compound of two or more subunit amino acids, amino acid analogs, or other peptidomimetics. The subunits may be linked by peptide bonds or by other bonds, for example ester, ether, etc. As used herein, the term "amino acid" refers to either natural and/or unnatural or synthetic amino acids, including glycine and both the D or L optical isomers, and amino acid analogs and peptidomimetics. A peptide of three or more amino acids is commonly called an oligopeptide if the peptide chain is short. If the peptide chain is long, the peptide is typically called a polypeptide or a protein.

"Operably linked" refers to an arrangement of elements wherein the components so described are configured so as to perform their usual function. Thus, a given promoter that is operably linked to a coding sequence (e.g., a reporter expression cassette) is capable of effecting the expression of the coding sequence when the proper enzymes are present. The promoter or other control elements need not be contiguous with the coding sequence, so long as they function to direct the expression thereof. For example, intervening untranslated yet transcribed sequences can be present between the promoter sequence and the coding sequence and the promoter sequence can still be considered "operably linked" to the coding sequence.

"Recombinant" as used herein to describe a nucleic acid molecule means a polynucleotide of genomic, cDNA, semisynthetic, or synthetic origin which, by virtue of its origin or manipulation: (1) is not associated with all or a portion of the polynucleotide with which it is associated in nature; and/or (2) is linked to a polynucleotide other than that to which it is linked in nature. The term "recombinant" as used with respect to a protein or polypeptide means a polypeptide produced by expression of a recombinant polynucleotide. "Recombinant host cells," "host cells," "cells," "cell lines," "cell cultures," and other such

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terms denoting procaryotic microorganisms or eucaryotic cell lines cultured as unicellular entities, are used interchangeably, and refer to cells which can be, or have been, used as recipients for recombinant vectors or other transfer DNA, and include the progeny of the original cell which has been transfected. It is understood that the progeny of a single parental cell may not necessarily be completely identical in morphology or in genomic or total DNA complement to the original parent, due to accidental or deliberate mutation. Progeny of the parental cell which are sufficiently similar to the parent to be characterized by the relevant property, such as the presence of a nucleotide sequence encoding a desired peptide, are included in the progeny intended by this definition, and are covered by the above terms.

An "isolated polynucleotide" molecule is a nucleic acid molecule separate and discrete from the whole organism with which the molecule is found in nature; or a nucleic acid molecule devoid, in whole or part, of sequences normally associated with it in nature; or a sequence, as it exists in nature, but having heterologous sequences (as defined below) in association therewith.

Techniques for determining nucleic acid and amino acid "sequence identity" also are known in the art. Typically, such techniques include determining the nucleotide sequence of the mRNA for a gene and/or determining the amino acid sequence encoded thereby, and comparing these sequences to a second nucleotide or amino acid sequence. In general, "identity" refers to an exact nucleotide-to-nucleotide or amino acid-to-amino acid correspondence of two polynucleotides or polypeptide sequences, respectively. Two or more sequences (polynucleotide or amino acid) can be compared by determining their "percent identity." The percent identity of two sequences, whether nucleic acid or amino acid sequences, is the number of exact matches between two aligned sequences divided by the length of the shorter sequences and multiplied by 100. An approximate alignment for nucleic acid sequences is provided by the local homology algorithm of Smith and Waterman, Advances in Applied Mathematics 2:482-489 (1981). This algorithm can be applied to amino acid sequences by using the scoring matrix developed by Dayhoff, Atlas of Protein Sequences and Structure, M.O. Dayhoff ed., 5 suppl. 3:353-358, National

Biomedical Research Foundation, Washington, D.C., USA, and normalized by Gribskov, Nucl. Acids Res. 14(6):6745-6763 (1986). An exemplary implementation of this algorithm to determine percent identity of a sequence is provided by the Genetics Computer Group (Madison, WI) in the "BestFit" utility application. The default parameters for this method are described in the Wisconsin Sequence Analysis Package Program Manual, Version 8 (1995) (available from Genetics Computer Group, Madison, WI). A preferred method of establishing percent identity in the context of the present invention is to use the MPSRCH package of programs copyrighted by the University of Edinburgh, developed by John F. Collins and Shane S. Sturrok, and distributed by IntelliGenetics, Inc. (Mountain View, CA). From this suite of packages the Smith-Waterman algorithm can be employed where default parameters are used for the scoring table (for example, gap open penalty of 12, gap extension penalty of one, and a gap of six). From the data generated the "Match" value reflects "sequence identity." Other suitable programs for calculating the percent identity or similarity between sequences are generally known in the art, for example, another alignment program is BLAST, used with default parameters. For example, BLASTN and BLASTP can be used using the following default parameters: genetic code = standard; filter = none; strand = both; cutoff = 60; expect = 10; Matrix = BLOSUM62; Descriptions = 50 sequences; sort by = HIGH SCORE; Databases = non-redundant, GenBank + EMBL + DDBJ + PDB + GenBank CDS translations + Swiss protein + Spupdate + PIR. Details of these programs can be found at the following internet address: http://www.ncbi.nlm.gov/cgi-bin/BLAST. When claiming sequences relative to sequences of the present invention, the range of desired degrees of sequence identity is approximately 80% to 100% and integer values therebetween. Typically the percent identities between the disclosed sequences and the claimed sequences are at least 80-82%, 85-90%, preferably 92%, more preferably 95%, and even more preferably 98% sequence identity to the reference sequence (i.e., the sequences of the present invention).

Alternatively, the degree of sequence similarity between polynucleotides can be determined by hybridization of polynucleotides under conditions that form stable duplexes between homologous regions, followed by digestion with single-stranded-specific

nuclease(s), and size determination of the digested fragments. Two DNA, or two polypeptide sequences are "substantially homologous" to each other when the sequences exhibit at least about 80%-85%, preferably at least about 85%-90%, more preferably at least about 90%-95%, and most preferably at least about 95%-98% sequence identity over a defined length of the molecules, as determined using the methods above. As used herein, substantially homologous also refers to sequences showing complete identity to the specified DNA or polypeptide sequence. DNA sequences that are substantially homologous can be identified in a Southern hybridization experiment under, for example, stringent conditions, as defined for that particular system. Defining appropriate hybridization conditions is within the skill of the art. See, e.g., Sambrook et al., *supra*; *DNA Cloning*, *supra*; *Nucleic Acid Hybridization*, *supra*.

Two nucleic acid fragments are considered to "selectively hybridize" as described herein. The degree of sequence identity between two nucleic acid molecules affects the efficiency and strength of hybridization events between such molecules. A partially identical nucleic acid sequence will at least partially inhibit a completely identical sequence from hybridizing to a target molecule. Inhibition of hybridization of the completely identical sequence can be assessed using hybridization assays that are well known in the art (e.g., Southern blot, Northern blot, solution hybridization, or the like, see Sambrook, et al., *Molecular Cloning: A Laboratory Manual*, Second Edition, (1989) Cold Spring Harbor, N.Y.). Such assays can be conducted using varying degrees of selectivity, for example, using conditions varying from low to high stringency. If conditions of low stringency are employed, the absence of non-specific binding can be assessed using a secondary probe that lacks even a partial degree of sequence identity (for example, a probe having less than about 30% sequence identity with the target molecule), such that, in the absence of non-specific binding events, the secondary probe will not hybridize to the target.

When utilizing a hybridization-based detection system, a nucleic acid probe is chosen that is complementary to a target nucleic acid sequence, and then by selection of appropriate conditions the probe and the target sequence "selectively hybridize," or bind, to each other to form a hybrid molecule. A nucleic acid molecule that is capable of hybridizing

selectively to a target sequence under "moderately stringent" typically hybridizes under conditions that allow detection of a target nucleic acid sequence of at least about 10-14 nucleotides in length having at least approximately 70% sequence identity with the sequence of the selected nucleic acid probe. Stringent hybridization conditions typically allow detection of target nucleic acid sequences of at least about 10-14 nucleotides in length having a sequence identity of greater than about 90-95% with the sequence of the selected nucleic acid probe. Hybridization conditions useful for probe/target hybridization where the probe and target have a specific degree of sequence identity, can be determined as is known in the art (see, for example, Nucleic Acid Hybridization: A Practical Approach, editors B.D. Hames and S.J. Higgins, (1985) Oxford; Washington, DC; IRL Press).

With respect to stringency conditions for hybridization, it is well known in the art that numerous equivalent conditions can be employed to establish a particular stringency by varying, for example, the following factors: the length and nature of probe and target sequences, base composition of the various sequences, concentrations of salts and other hybridization solution components, the presence or absence of blocking agents in the hybridization solutions (e.g., formamide, dextran sulfate, and polyethylene glycol), hybridization reaction temperature and time parameters, as well as, varying wash conditions. The selection of a particular set of hybridization conditions is selected following standard methods in the art (see, for example, Sambrook, et al., Molecular Cloning: A Laboratory Manual, Second Edition, (1989) Cold Spring Harbor, N.Y.).

A "vector" is capable of transferring gene sequences to target cells. Typically, "vector construct," "expression vector," and "gene transfer vector," mean any nucleic acid construct capable of directing the expression of a gene of interest and which can transfer gene sequences to target cells. Thus, the term includes cloning, and expression vehicles, as well as integrating vectors.

"Nucleic acid expression vector" or "expression cassette" refers to an assembly which is capable of directing the expression of a sequence or gene of interest. The nucleic acid expression vector includes a promoter which is operably linked to the sequences or gene(s) of interest. Other control elements may be present as well. Expression cassettes

described herein may be contained within a plasmid construct. In addition to the components of the expression cassette, the plasmid construct may also include a bacterial origin of replication, one or more selectable markers, a signal which allows the plasmid construct to exist as single-stranded DNA *(e.g.,* a M13 origin of replication), a multiple cloning site, and a "mammalian" origin of replication (e.g., a SV40 or adenovirus origin of replication).

An "expression cassette" comprises any nucleic acid construct capable of directing the expression of a gene/coding sequence of interest. Such cassettes can be constructed into a "vector," "vector construct," "expression vector," or "gene transfer vector," in order to transfer the expression cassette into target cells. Thus, the term includes cloning and expression vehicles, as well as viral vectors.

"Luciferase," unless stated otherwise, includes prokaryotic and eukaryotic luciferases, as well as variants possessing varied or altered optical properties, such as luciferases that produce different colors of light (e.g., Kajiyama, N., and Nakano, E., *Protein Engineering* **4**(6):691-693 (1991)).

"Light-generating" is defined as capable of generating light through a chemical reaction or through the absorption of radiation.

A "light generating protein" or "light-emitting protein" is a protein capable of generating light in the visible spectrum (between approximately 350 nm and 800 nm). Examples include bioluminescent protiens such as luciferases, *e.g.,* bacterial and firefly luciferases, as well as fluorescent proteins such as green fluorescent protein (GFP).

"Light" is defined herein, unless stated otherwise, as electromagnetic radiation having a wavelength of between about 300 nm and about 1100 nm.

"Animal" as used herein typically refers to a non-human mammal, including, without limitation, farm animals such as cattle, sheep, pigs, goats and horses; domestic mammals such as dogs and cats; laboratory animals including rodents such as mice, rats and guinea pigs; birds, including domestic, wild and game birds such as chickens, turkeys and other gallinaceous birds, ducks, geese, and the like. The term does not denote a particular age. Thus, both adult and newborn individuals are intended to be covered.

A "transgenic animal" refers to a genetically engineered animal or offspring of genetically engineered animals. A transgenic animal usually contains material from at least one unrelated organism, such as from a virus, plant, or other animal. The "non-human animals" of the invention include vertebrates such as rodents, non-human primates, sheep, dogs, cows, amphibians, birds, fish, insects, reptiles, etc. The term "chimeric animal" is used to refer to animals in which the heterologous gene is found, or in which the heterologous gene is expressed in some but not all cells of the animal.

"Analyte" as used herein refers to any compound or substance whose effects (*e.g.*, induction or repression of a specific promoter) can be evaluated using the test animals and methods of the present invention. Such analytes include, but are not limited to, chemical compounds, pharmaceutical compounds, polypeptides, peptides, polynucleotides, and polynucleotide analogs. Many organizations (*e.g.*, the National Institutes of Health, pharmaceutical and chemical corporations) have large libraries of chemical or biological compounds from natural or synthetic processes, or fermentation broths or extracts. Such compounds/analytes can be employed in the practice of the present invention.

As used herein, the term "positive selection marker" refers to a gene encoding a product that enables only the cells that carry the gene to survive and/or grow under certain conditions. For example, plant and animal cells that express the introduced neomycin resistance (Neo^r) gene are resistant to the compound G418. Cells that do not carry the Neo^r gene marker are killed by G418. Other positive selection markers will be known to those of skill in the art. Typically, positive selection markers encode products that can be readily asssayed. Thus, positive selection markers can be used to determine whether a particular DNA construct has been introduced into a cell, organ or tissue.

"Negative selection marker" refers to gene encoding a product which can be used to selectively kill and/or inhibit growth of cells under certain conditions. Non-limiting examples of negative selection inserts include a herpes simplex virus (HSV)-thymidine kinase (TK) gene. Cells containing an active HSV-TK gene are incapable of growing in the presence of gangcylovir or similar agents. Thus, depending on the substrate, some gene products can act as either positive or negative selection markers.

The term "homologous recombination" refers to the exchange of DNA fragments between two DNA molecules or chromatids at the site of essentially identical nucleotide sequences. It is understood that substantially homologous sequences can accommodate insertions, deletions, and substitutions in the nucleotide sequence. Thus, linear sequences of nucleotides can be essentially identical even if some of the nucleotide residues do not precisely correspond or align (see, above).

A "knock-out" mutation refers to partial or complete loss of expression of at least a portion the target gene. Examples of knock-out mutations include, but are not limited to, gene-replacement by heterologous sequences, gene disruption by heterologous sequences, and deletion of essential elements of the gene (e.g., promoter region, portions of a coding sequence). A "knock-out" mutation is typically identified by the phenotype generated by the mutation.

A "single-copy gene" as used herein refers to a gene represented in an organism's genome only by a single copy at a particular chromosomal locus. Accordingly, a diploid organism has two copies of the gene and both copies occur at the same chromosomal location.

A "gene" as used in the context of the present invention is a sequence of nucleotides in a genetic nucleic acid (chromosome, plasmid, etc.) with which a genetic function is associated. A gene is a hereditary unit, for example of an organism, comprising a polynucleotide sequence (e.g., a DNA sequence for mammals) that occupies a specific physical location (a "gene locus" or "genetic locus") within the genome of an organism. A gene can encode an expressed product, such as a polypeptide or a polynucleotide (e.g., tRNA). Alternatively, a gene may define a genomic location for a particular event/function, such as the binding of proteins and/or nucleic acids (e.g., phage attachment sites), wherein the gene does not encode an expressed product. Typically, a gene includes coding sequences, such as, polypeptide encoding sequences, and non-coding sequences, such as, promoter sequences, poly-adenlyation sequences, transcriptional regulatory sequences (e.g., enhancer sequences). Many eucaryotic genes have "exons" (coding sequences) interrupted

by "introns" (non-coding sequences). In certain cases, a gene may share sequences with another gene(s) (e.g., overlapping genes).

"Isogenic" means two or more organisms or cells that are considered to be genetically identical. "Substantially isogenic" means two or more organisms or cells wherein, at the majority of genetic loci (e.g., greater than 99.000%, preferably more than 99.900%, more preferably greater than 99.990%, even more preferably greater than 99.999%), there exists genetic identity between the organisms or cells being compared. In the context of the present invention, two organisms (for example, mice) are considered to be "substantially isogenic" if, for example, inserted transgenes are the primary differences between the genetic make-up of the mice being compared. Further, if, for example, the genetic backgrounds of the mice being compared are the same with the exception that one of the mice has one or several defined mutation(s) (for example, affecting coat color), then these mice are considered to be substantially isogenic. An example of two strains of substantially isogenic mice are C57BL/6 and C57BL/6-Tyr C2j/+.

A "pseudogene" as used herein, refers to a type of gene sequence found in the genomes, typically, of eucaryotes, where the sequence closely resembles a known functional gene, but differs in that the pseudogene is non-functional. For example, the pseudogene sequence may contain several stop codons in what would correspond to an open reading frame in the functional gene. Pseudogenes can also have deletions or insertions relative to their corresponding functional gene. If, for example, in a genome there is a functional gene and a related pseudogene, the functional gene is considered to be a single-copy gene (accordingly, the pseudogene is considered to be single-copy as well).

A "non-essential gene" refers to a gene whose deletion, disruption, elimination, reduction of gene function, or mutation is non-lethal, and does not obviously adversely affect the organisms' ability to mature and reproduce. A "non-essential gene with no phenotype" refers to a non-essential gene whose deletion, disruption, elimination, reduction of gene function or mutation has no deleterious effect on the organism. Typically there are no phenotypically reflected gene dosage effects associated with modification of a non-essential gene with no phenotype -- for example, deletion, disruption or mutation of both

copies of a non-essential gene with no phenotype in a diploid organism has essentially the same effect as deletion, disruption, or mutation of one of the two copies present in the diploid organism. In the context of the present invention, a non-essential gene is typically one whose function has been eliminated (e.g., by a deletion mutation) and such elimination of function was non-lethal and the organism developed, matured, and was able to reproduce.

The "native sequence" or "wild-type sequence" of a gene is the polynucleotide sequence that comprises the genetic locus corresponding to the gene, e.g., all regulatory and open-reading frame coding sequences required for expression of a completely functional gene product as they are present in the wild-type genome of an organism. The native sequence of a gene can include, for example, transcriptional promoter sequences, translation enhancing sequences, introns, exons, and poly-A processing signal sites. It is noted that in the general population, wild-type genes may include multiple prevalent versions that contain alterations in sequence relative to each other and yet do not cause a discernible pathological effect. These variations are designated "polymorphisms" or "allelic variations."

By "replacement sequence" is meant a polynucleotide sequence that is substituted for at least a portion of the native or wild-type sequence of a gene.

"Linear vector" or "linearized vector," as used herein, is a vector having two ends. For example, circular vectors, such as plasmids, can be linearized by digestion with a restriction endonuclease that cuts at a single site in the plasmid. Preferably, the targeting vectors described herein are linearized such that the ends are not within the targeting sequences.

General Overview

In one aspect, the present invention relates to vector constructs, cells containing the constructs, methods of screening compounds, and methods of creating transgenic animals to be used, for example, as screening or test systems. Methods of using the constructs, cells, and transgenic animals of the present invention include, but are not limited to, studies involving tumor growth and other disease conditions. Exemplary promoters useful in the practice of the present invention include mouse VEGFR-2 and mouse Tie2. In one

embodiment, the present invention relates to novel promoters for the mouse VEGFR-2 receptor gene, nucleic acid constructs comprising such promoters operatively linked to genes encoding a gene product, such as, a reporter, a protein, polypeptide, hormone, ribozyme, or antisense RNA, recombinant cells comprising such nucleic acid constructs, screening for therapeutic drugs using such cells (e.g., screening for compounds that modulate VEGFR-2-mediated angiogenesis), and endothelial tissue-specific gene expression using these novel promoter sequences.

In yet another aspect of the present invention, transgenic, non-human mammals are constructed where a single-copy, non-essential gene is replaced by a reporter expression cassette, preferably a gene encoding a light-generating protein, such as a luciferase-encoding gene, operably linked to a promoter. A variety of promoters are useful in the practice of the present invention, for example, promoters derived from genes associated with tumorigenesis or angiogenesis. Thus, an exemplary promoter can be one that is associated with proteins induced during tumorigenesis, for instance in the presence of tumor generating compounds or of tumors themselves. In this way, expression of the reporter cassette is induced in the animal when, for example, tumors are present, and progression of the tumor can be evaluated by non-invasive imaging methods using the whole animal. Another exemplary promoter is one that is derived from a gene associated with angiogenesis. Because the promoter is linked to a reporter such as luciferase, non-invasive monitoring of the progression of angiogenesis is possible. Various forms of the different embodiments of the invention, described herein, may be combined.

Non-invasive imaging and/or detecting of light-emitting conjugates in mammalian subjects was described in U.S. Patent No. 5,650,135, by Contag, et al., issued 22 July 1997, and herein incorporated by reference. This imaging technology can be used in the practice of the present invention in view of the teachings of the present specification. In the imaging method, the conjugates contain a biocompatible entity and a light-generating moiety. Biocompatible entities include, but are not limited to, small molecules such as cyclic organic molecules; macromolecules such as proteins; microorganisms such as viruses, bacteria, yeast and fungi; eukaryotic cells; all types of pathogens and pathogenic substances; and

particles such as beads and liposomes. In another aspect, biocompatible entities may be all or some of the cells that constitute the mammalian subject being imaged, for example, cells carrying the vector constructs of the present invention expressing a reporter expression cassette.

Light-emitting capability is conferred on the biocompatible entities by the conjugation of a light-generating moiety. Such moieties include fluorescent molecules, fluorescent proteins, enzymatic reactions giving off photons and luminescent substances, such as bioluminescent proteins. In the context of the present invention, light emitting capability is typically confered on target cells by having at least one copy of a light-generating protein, *e.g.,* a luciferase, present. In preferred embodiments, luciferase is operably linked to appropriate control elements which can facilitate expression of a polypeptide having luciferase activity. Substrates of luciferase can be endogenous to the cell or applied to the cell or system (e.g., injection into a transgenic mouse, having cells carrying a luciferase construct, of a suitable substrate for the luciferase, for example, luciferin). The conjugation may involve a chemical coupling step, genetic engineering of a fusion protein, or the transformation of a cell, microorganism or animal to express a light-generating protein.

Targeting Constructs

The targeting cassettes described herein typically include the following components: (1) a suitable vector backbone; (2) a polynucleotide encoding a light generating protein, (3) a promoter operably linked to the luciferase-encoding gene, wherein the promoter is heterologous to the coding sequences of the light generating protein; (4) a sequence encoding a positive selection marker; (5) insertion sites flanking the sequence encoding the positive selection marker and the polynucleotide encoding a light generating protein gene, for insertion of sequences which target a single-copy, non-essential chromosomal gene; and, optionally, (6) a sequence encoding a negative selection marker. Exemplary targeting constructs are shown in Figures 3B, 13 and 18 and described in Examples 1-3.

Suitable vector backbones generally include an F1 origin of replication; a colE1 plasmid-derived origin of replication; polyadenylation sequence(s); sequences encoding antibiotic resistance (*e.g.*, ampicillin resistance) and other regulatory or control elements. Non-limiting examples of appropriate backbones include: pBluescriptSK (Stratagene, La Jolla, CA); pBluescriptKS (Stratagene, La Jolla, CA) and other commercially available vectors.

In one aspect of the invention, the light generating protein is luciferase. Luciferase coding sequences useful in the practice of the present invention include sequences obtained from *lux* genes (procaryotic genes encoding a luciferase activity) and *luc* genes (eucaryotic genes encoding a luciferase activity). A variety of luciferase encoding genes have been identified including, but not limited to, the following: B.A. Sherf and K.V. Wood, U.S. Patent No. 5,670,356, issued 23 September 1997; Kazami, J., et al., U.S. Patent No. 5,604,123, issued 18 February 1997; S. Zenno, et al, U.S. Patent No. 5,618,722; K.V. Wood, U.S. Patent No. 5,650,289, issued 22 July 1997; K.V. Wood, U.S. Patent No. 5,641,641, issued 24 June 1997; N. Kajiyama and E. Nakano, U.S. Patent No. 5,229,285, issued 20 July 1993; M.J. Cormier and W.W. Lorenz, U.S. Patent No. 5,292,658, issued 8 March 1994; M.J. Cormier and W.W. Lorenz, U.S. Patent No. 5,418,155, issued 23 May 1995; de Wet, J.R., et al, *Molec. Cell. Biol.* **7**:725-737, 1987; Tatsumi, H.N., et al, *Biochim. Biophys. Acta* 1131:161-165, 1992; and Wood, K.V., et al, *Science* 244:700-702, 1989; all herein incorporated by reference. Eukaryotic luciferase catalyzes a reaction using luciferin as a luminescent substrate to produce light, whereas prokaryotic luciferase catalyzes a reaction using an aldehyde as a luminescent substrate to produce light.

Wild-type firefly luciferases typically have an emission maxima at about 550 nm. Numerous variants with differing emission maxima have also been studied. For example, Kajiyama and Nakano (*Protein Eng.* **4**(6):691-693, 1991; U.S. Patent No. 5,330,906, issued 19 July 1994, herein incorporated by reference) teach five variant firefly luciferases generated by single amino acid changes to the *Luciola cruciata* luciferase coding sequence. The variants have emission peaks of 558 nm, 595 nm, 607 nm, 609 nm and 612 nm. A yellow-green luciferase with an emission peak of about 540 nm is commerically available

from Promega, Madison, WI under the name pGL3. A red luciferase with an emission peak of about 610 nm is described, for example, in Contag et al. (1998) *Nat. Med.* 4:245-247 and Kajiyama et al. (1991) *Prot. Eng.* 4:691-693.

Positive selection markers include any gene which a product that can be readily asssayed. Examples include, but are not limited to, a hprt gene (Littlefield, J. W., Science 145:709-710 (1964), herein incorporated by reference), a xanthine-guanine phosphoribosyltransferase (gpt) gene, or an adenosine phosphoribosyltransferase (aprt) gene (Sambrook et al., *supra*), a thymidine kinase gene (i.e "TK") and especially the TK gene of herpes simplex virus (Giphart-Gassler, M. et al., Mutat. Res. 214:223-232 (1989) herein incorporated by reference), a nptII gene (Thomas, K. R. et al., Cell 51:503-512 (1987); Mansour, S. L. et al., Nature 336:348-352 (1988), both references herein incorporated by reference), or other genes which confer resistance to amino acid or nucleoside analogues, or antibiotics, etc, for example, gene sequences which encode enzymes such as dihydrofolate reductase (DHFR) enzyme, adenosine deaminase (ADA), asparagine synthetase (AS), hygromycin B phosphotransferase, or a CAD enzyme (carbamyl phosphate synthetase, aspartate transcarbamylase, and dihydroorotase). Addition of the appropriate substrate of the positive selection marker can be used to determine if the product of the positive selection marker is expressed, for example cells which do not express the positive selection marker nptII, are killed when exposed to the substrate G418 (Gibco BRL Life Technology, Gaithersburg, MD).

The targeting vector typically contains insertion sites for inserting targeting sequences (*e.g.*, sequences that are substantially homologous to the target sequences in the host genome where integration of the targeting vector/expression cassette is desired). These insertion sites are preferably included such that there are two sites, one site on either side of the sequences encoding the positive selection marker, luciferase and the promoter. Insertion sites are, for example, restriction endonuclease recognition sites, and can, for example, represent unique restriction sites. In this way, the vector can be digested with the appropriate enzymes and the targeting sequences ligated into the vector.

Optionally, the targeting construct can contain a polynucleotide encoding a negative selection marker. Suitable negative selection markers include, but are not limited to, HSV-tk (see, e.g., Majzoub et al. (1996) *New Engl. J. Med.* 334:904-907 and U.S. Patent No. 5,464,764), as well as genes encoding various toxins including the diphtheria toxin, the tetanus toxin, the cholera toxin and the pertussis toxin. A further negative selection marker gene is the hypoxanthine-guanine phosphoribosyl transferase (HPRT) gene for negative selection in 6-thioguanine.

Exemplary promoters and single-copy, non-essential genes for use in the vector constructs and methods of the present invention are described below.

Promoters

The targeting constructs and transgenic animals described herein contain a sequence encoding a luciferase gene operably linked to a promoter. The promoter may be from the same species as the transgenic animal (*e.g.*, mouse promoter used in construct to make transgenic mouse) or from a different species (*e.g.*, human promoter used in construct to make transgenic mouse). The promoter can be derived from any gene of interest. In one embodiment of the present invention, the promoter is derived from a gene whose expression is induced during angiogenesis, for example pathogenic angiogenesis like tumor development. Thus, when a tumor begins to develop in a transgenic animal carrying a vector construct of the present invention, the promoter is induced and the animal expresses luciferase, which can then be monitored *in vivo*.

Exemplary promoters for use in the present invention are selected such that they are functional in a cell type and/or animal into which they are being introduced. Exemplary promoters include, but are not limited to, promoters obtained from the following mouse genes: vascular endothelial growth factor (VEGF) (VEGF promoter described in U.S. Patent No. 5,916,763; Shima et al. (1996) *J. Bio. Chem.* 271:3877-3883; sequence available on NCBI under accession number U41383); VEGFR2, also known as Flk-1, (VEGFR-2 promoter described, for example, in Rönicke et al. (1996) *Circ. Res.* 79:277-285; Patterson et al. (1995) *J. Bio. Chem.* 270:23111-23118; Kappel et al. (1999) *Blood* 93:4282-4292;

sequence available as accession number X89777 of NCBI database); Tie2, also known as Tek (Tie2 promoter described, for example, in Fadel et al. (1998) *Biochem. J.* 338:335-343; Schlaeger et al. (1995) *Develop.* 121:1089-1098; Schlager et al. (1997) *PNAS USA* 94:3058-3063). VEGF is a specific mitogen for EC *in vitro* and a potent angiogenic factor *in vivo*. In a tumorigenesis study, it was shown that VEGF was critical for the initial subcutaneous growth of T-47D breast carcinoma cells transplanted into nude mice, whereas other angiogenic factors, such as, bFGF can compensate for the loss of VEGF after the tumors have reached a certain size (Yoshiji, H., et al.,1997 Cancer Research 57: 3924-28). VEGF is a major mediator of aberrant EC proliferation and vascular permeability in a variety of human pathologic situation, such as, tumor angiogenesis, diabetic retinopathy and rheumatoid arthritis (Benjamin LE, et al.,1997 PNAS 94: 8761-66; Soker, S., et al.,1998 Cell 92: 735-745). VEGF is synthesized by tumor cells *in vivo* and accumulates in nearby blood vessels. Because leaky tumor vessels initiate a cascade of events, which include plasma extravasation and which lead ultimately to angiogenesis and tumor stroma formation, VEGF plays a pivotal role in promoting tumor growth (Dvorak, H.F., et al., 1991 J Exp Med 174:1275-8). VEGF expression was upregulated by hypoxia (Shweiki, D., et al., 1992 Nature 359: 843-5). VEGF is also upregulated by overexpression of v-Src oncogene (Mukhopadhyay. D., et al.,1995 Cancer Res. 15: 6161-5), c-SRC (Mukhopadhyay, D., et al., 1995 Nature 375: 577-81), and mutant ras oncogene (Plate, K.H., et al., 1992 Nature 359: 845-8). The tumor suppressor p53 downregulates VEGF expression (Mukhopadhyay. D., et al.,1995 Cancer Res. 15: 6161-5).

A number of cytokines and growth factors, including PGF and TPA (Grugel, S., et al., 1995 J. Biological Chem. 270: 25915-9), EGF, TGF-b, IL-1, IL-6 induce VEGF mRNA expression in certain type of cells (Ferrara, N., et al., 1997 Endocr. Rev. 18: 4-25). Kaposi's sarcoma-associated herpesvirus (KSHV) encoded a G-protein-coupled receptor, a homolog of IL-8 receptor, can activate JNK/SAPK and p38MAPK and increase VEGF production, thus causing cell transformation and tumorigenicity (Bais, C., et al., Nature 1998 391:86-9). VEGF overexpression in skin of transgenic mice induces angiogenesis,

vascularhyperpermeability and accelerated tumor development (Larcher, F., et al., Oncogene 1998 17:303-11).

VEGF-B (cDNA sequences available on databases) is a mitogen for EC and may be involved in angiogenesis in muscle and heart (Olofsson, B., et al., 1996 Proc Natl Acad Sci U S A 93:2576-81). Shown *in vitro*, binding of VEGF-B to its receptor VEGFR-1 leads to increased expression and activity of urokinase type plasminogen activator and plasminogen activator inhibitor, suggesting a role for VEGF-B in the regulation of extracellular matrix degradation, cell adhesion, and migration (Olofsson, B., et al., 1998 Proc Natl Acad Sci U S A 95:11709-14).

VEGF-C (see, e.g., U.S. Patent No. 5,916,763 and Shima et al., *supra*) may regulate angiogenesis of lymphatic vasculature, as suggested by the pattern of VEGF-C expression in mouse embryos (Kukk, E., et al., 1996 Development 122: 3829-37). Although VEGF-C is also a ligand for VEGFR-2, the functional significance of this potential interaction is unknown. Overexpression of VEGF-C in the skin of transgenic mice resulted in lymphatic, but not vascular, endothelial proliferation and vessel enlargement, suggesting the major function of VEGF-C is through VEGFR-3 rather than VEGFR-2 (Jeltsch M, et al., 1997 Science 276:1423-5). Shown by the CAM assay, VEGF and VEGF-C are specific angiogenic and lymphangiogenic growth factors, respectively (Oh, S.J., et al., (1997) *Devel. Biol.* 188: 96-109). VEGF-C overexpression in the skin of transgenic mice resulted in lymphatic, but not vascular, endothelial proliferation and vessel enlargement (Jeltsch M, et al., 1997 Science 276:1423-5).

VEGF-D (cDNA sequences available on databases) is a mitogen for EC. Given that VEGF-D can also activate VEGFR-3. it is possible that VEGF-D could be involved in the regulation of growth and/or differentiation of lymphatic endothelium (Achen, M.G., et al., 1998 Proc Natl Acad Sci U S A 95: 548-53). VEGF-D is induced by transcription factor c-Fos in mouse (Orlandini, M., 1996 PNAS 93: 11675-80).

VEGFR-1 signaling pathway may regulate normal endothelial cell-cell or cell matrix interactions during vascular development, as suggested by the knockout study (Fong, G.H., et al., 1995 Nature 376: 65-69). Although VEGFR-1 has a higher affinity to VEGF than

VEGFR-2, it does not transduce the mitogenic signals of VEGF in ECs (Soker, S., et al.,1998 Cell 92: 735-745). VEGFR-2 (see, e.g., Rönicke et al., Patterson et al., Kappel et al. (1999), *supra*) appears to be the major transducer of VEGF signals in EC that result in chemotaxis, mitogenicity and gross morphological changes in target cells (Soker, S., et al.,1998 Cell 92: 735-745). The cloning and sequencing of the 4.5 kb VEGFR2 promoter region is described herein (Example 3).

VEGFR-3 has an essential role in the development of the embryonic cardiovascular system before the emergence of lymphatic vessels, as shown by the knockout study (Dumont, D.J., et al., 1998 Science 282: 946-949). Neuropillin-1 (see, e.g., Soker et al. (1998) *Cell* 92:735-745) is a receptor for VEGF165. It can enhance the binding of VEGF165 to VEGFR-2 and VEGF165 mediated chemotaxis (Soker, S., et al.,1998 Cell 92: 735-745). Neuropillin1 overexpression in transgenic mice resulted in embryonic lethality. The embryos possessed excess capillaries and blood vessels. Dilated vessels and hemorrhage were also observed (Kitsukawa, T., et al., 1995 Development 121: 4309-18).

Further promoters of interest include, but are not limited to, the following. Ang2 is expressed only at predominant vascular remodeling sites, such as ovary, placenta, uterus (Maisonpierre, P.C., et al., 1997 Science 277: 55-60). In glioblastoma angiogenesis, Ang2 is found to be expressed in endothelial cells of small blood vessel and capilaries while Ang1 is expressed in glioblastoma tumor cells (Stratmann, A., 1998 Am J Pathol 153: 1459-66). Ang2 is up-regulated in bovine microvascular endothelial by VEGF, bFGF, cyrokines, hypoxia (Mandriota, S.J., 1998 Circ Res 83: 852-9). Ang2 transgenic overexpression disrupts angiogenesis, and is embryonic lethal (Maisonpierre, P.C., et al., 1997 Science 277: 55-60). Ang1 is widely expressed, less aboundant in heart and liver (Maisonpierre, P.C., et al., 1997 Science 277: 55-60). Ang1 is expressed in mesenchymal cells and may up-regulate the expression of Tie2 in the endothelial cells (Suri,C., et al., 1996 Cell 87: 1171-1180). Ang1 overexpression in the skin of transgenic mice produces larger, more numerous, and more highly branched vessels (Suri, C., et al., Science 1998 282:468-71).

Tie2 (see, e.g., Fadel et al.; Schlaeger et al. (1995), and Schlager et al. (1997), *supra*) is edothelial cell specific, up-regulated during wound healing, follicle maturation (Puri,

M.C., et al., 1995 EMBO J 14: 5884-91) and pathologic angiogenesis (Kaipainen, A., 1994 Cancer Research 54: 6571-77), such as, glioblastoma (Stratmann, A., 1998 Am J Pathol 153: 1459-66). Tie2 is also expressed in non-proliferating adult endothelium and edothelial cell lines (Dumont, D.J., et al. (1994) *Genes & Develop.* 8:1897-1909). A Tie2 activating mutation causes vascular dysmorphorgenesis (Vikkula M, et al., 1996 Cell 87: 1181-1190). Tie2 mutant overexpression in transgenic mice is embryonic lethal (Dumont, D.J., et al., *supra*). The cloning and sequencing of the 7.1 kb promoter region of Tie2 is described herein (Example 3).

Other promoters useful in the practice of the present invention include, by way of example, promoters derived from the sequences encoding the following polypeptide products: PTEN (dual specificity phosphatase); BAI (brain-specific angiogenesis inhibitor); KAI1 (KANGAI 1); catenin beta-1 (cadherin-associated protein, beta); COX2 (PTGS2 cyclooxygenase 2, a.k.a. prostaglandin-endoperoxide synthase 2); MMP2 (72 kDa Type IV-A collagenase); MMP9 (92 kDa type IV-B collagenase); TIMP2 (tissue inhibitor of metalloproteinase 2); and TIMP3 (tissue inhibitor of metalloproteinase 3).

PTEN is a tumor suppressor gene and encodes a protein of 403 amino acids. (Li et al. (1997) *Science* 275:1943-1946; DiCristofano et al. (1998) *Nature Genet.* 19:348-355). Overexpression of PTEN has been shown to inhibit cell migration and it is postulated that this protein may function as a tumor suppressor by negatively regulating cell interactions with the extracellular matrix or by negatively regulating the PI3K/PKB/Akt signaling pathway. (Tamura et al. (1998) *Science* 280:1614-1617; Stambolic et al. (1998) *Cell* 95:29-29). Mutations in PTEN have been detected in cancer cell lines and in the germline of patients having Cowden disease, Lhermitte-Duclos disease and Bannayan-Zonana syndrome (diseases and syndromes which are characterized by hyperplastic/dysplastic changes in the prostate, skin and colon and which are associated with an increased risk of certain cancers, for example, breast cancer, prostate cancer and colon cancer). (Marsh et al. (1998) *Hum. Molec. Genet.* 7:507-515; Marsh et al. (1998) *J. Med. Genet.* 35:881-885; Nelen et al. (1997) *Hum. Molec. Genet.* 6:1383-1387).

BAI1 protein is predicted to be 1,584 amino acids in length and includes an extracellular domain, an intracellular domain and a 7-span transmembrane region similar to that of the secretin receptor. (Nishimori et al. (1997) *Oncogene* 15:245-2150). The extracellular region of BAI1 has a single Arg-Gly-Asp (RGD) motif recognized by integrins and also has five sequences corresponding to the thrombospondin type I (accession number 188060) repeats that can inhibit angiogenesis includes by basic fibroblast growth factor (bFGF, accession number 134920). Shiratsuchi et al. (1997) *Cytogenet. Cell Genet.* 79:103-108, cloned 2 other brain-specific angiogenesis inhibiting genes, designated BAI2 (accession number 602683) and BAI 3 (accession number 602684). Thus, it is postulated that members of this gene family may play a role in suppression of glioblastoma.

KAI1 encodes a 267 amino acid protein which is a member of the leukocyte surface glyoprotein family. The protein has 4 hydrophobobic transmembrane domains and 1 large extracellular hydrophilic domain with three potential N-glycosylation sites. (Dong et al. (1995) *Science* 268:884-886). Molecular analysis of KAI1 is described, for example, in Dong et al. (1997) *Genomics* 41:25-32. KAI1 is a tumor metastasis suppressor gene that is capable of inhibiting the metastatic process in experimental animals. Expression of KAI1 is downregulated during tumor progression of prostate, breast, lung, bladder and pancreatic cancers in humans, apparently at the transcriptional or postranscriptional level. Mashimo et al. (1998) *PNAS USA* 95:11307-11311, found that the tumor suppressor gene p53 can directly inactivate the KAI1 gene by interacting with the region 5' to the coding sequence, suggesting a direct relationship between p53 and KAI1.

Catenin beta-1 is an adherens junction (AJ) protein, which are critical for establishing and maintaining epithelial cell layers, for instance during embryogenesis, wound healing and tumor cell metastasis. Molecular analysis, including description of sequence homology to plakoglobin (accession number 173325), homology to the drosophila gene "armadillo" and interactions with Lef1/Tcf DNA binding proteins, is described, for example, in Nollet et al. (1996) *Genomics* 32:413-424; McCrea et al. (1991) *Science* 254:1359-1361 and Korinek et al. (1997) *Science* 275:1784-1787. In addition, studies by Korinek et al., *supra* and Morin et al.(1997) *Science* 275:1787-1790, have indicated that

APC (accession number 175100) negatively regulates catenin beta and that regulation of this protein is critical to the tumor suppressive effect of APC. Abnormally high levels of beta-catenin have been detected in certain human melanoma cell lines. (Rubinfeld et al. (1997) *Science* 275:1790-1792. Koch et al. (1999) *Cancer Res.* 59:269-273 report that childhood hepatoblastomas frequently carry a mutated degradation targeting box of the beta-catenin gene. Transgenic mice which express catenin beta under the control of an epidermal promoter undergo de novo hair morphogenesis and eventually these animals develop two types of tumors -- epithelioid cysts and trichofolliculomas. Gat et al. (1998) *Cell* 95:605-614.

COX2 encodes a cyclooxygenase and is a key regulator of prostaglandin synthesis. (Hla et al. (1992) *PNAS USA* 89:7384-7388; Jones et al. (1993) *J. Biol. Chem.* 268:9049-9054). In particular, COX2 is generally considered to be a mediator of inflammation and overexpression of COX2 in rat epithelial cells results in elevated levels of E-cadherin and Bcl2. (Tsujii & DuBois (1995) *Cell* 83:493-501). In co-cultures of endothelial cells and colon carcinoma cells, cells that overexpress COX2 produce prostaglandins, proangiogenic factors and stimulate both endothelial migration and tube formation. (Tsujii et al. (1998) *Cell* 93:705-716). Experiments conducted using APC knock-out mice have demonstrated that animals homozygous for a disrupted COX2 locus develop significantly more adenomatous polyps. (Oshima et al. (1996) *Cell* 87:803-809). COX-2 "knock out" mice develop severe nephropathy, are susceptible to peritonitis, exhibit reduced arachidonic acid-induced inflammation and exhibit reduced indomethacin-induced gastric ulceration. (Morham et al. (1995) *Cell* 83:473-482; Langenbach et al. (1995) *Cell* 83:483-492). Female mice that are deficient in cyclooxygenase 2 exhibit multiple reproductive failures. (Lim et al. (1997) *Cell* 91:197-208.

MMP2 is a metalloproteinase that specifically cleaves type IV collagen. A C-terminal fragment of MMP2, termed PEX, prevents normal biding to alpha-V/beta-3 and disrupts angiogenesis and tumor growth. (Brooks et al. (1998) *Cell* 92:391-400).

MMP9 is a collagenase secreted from normal skin fibroblasts. MMP9 null mice exhibit an abnormal pattern of skeletal growth plate vascularization and ossification. (Vu et al. (1998) *Cell* 93:411-422).

TIMP2 is a collagenase and appears to play a major role in modulating the activity of interstitial collagenase and a number of connective tissue metalloendoproteases. (Stetler-Stevenson et al. (1989) *J. Biol. Chem.* 264:17372-17378). Unlike TIMP1 and TIMP3, TIMP2 is not upregulated by TPA or TGF-beta. (Hammani et al. (1996) *J. Biol. Chem.* 271:25498-25505).

TIMP3 (Wilde et al. (1994) *DNA Cell Biol.* 13:711-718) is localized in the extracellular matrix in both its glycosylated and unglycosylated forms. Studies of mutant TIMP3 proteins have demonstrated that C-terminal trunctions do not bind to the extracellular matrix. (Langton et al. (1998) *J. Biol. Chem.* 273:16778-16781).

As one of skill in the art will appreciate in view of the teachings of the present specification, promoter sequences can be derived and isolated from known polypeptide sequences or from cDNA or genomic sequences, using method known in the art in view of the teachings herein, for example the promoter sequences of VEGFR2 and Tie2 were isolated and sequenced as described in Example 3 below. Another exemplary method of isolating promoter sequences using cDNA is via a GenomeWalker® kit, commercially available from Clontech (Palo Alto, CA), and described on page 27 of the 1997-1998 Clontech catalog.

Targeting Sequences: Non-Essential Genes

Central to the present invention is the fact that the targeting constructs contain "targeting" sequences (flanking, for example, the luciferase-encoding sequence and promoter) derived from a single-copy, non-essential gene. These targeting sequences in the construct act via homologous recombination to replace at least a portion of the non-essential gene in the genome with the light-generating protein-encoding (*e.g.*, luciferase-encoding) sequence operably linked to a promoter.

Non-limiting examples of targeting sequences for use in generating transgenic mice include sequences obtained from or derived from vitronectin, Fos B and galactin 3. A search of Mouse Knockout & Mutation Database (Genome Systems, Inc., St. Louis, MO) can be used to identify genes that have been knocked-out in mice where the generated knockout mice displayed no obvious defects. The chromosomal locus for all these genes can be used to target promoter-luciferase transgenes similar to what is described in Example 2. Single-copy, non-essential mouse genes identified in this manner include, but are not limited to, the following: Moesin (Msn), Doi Y., et al., J Biol Chem 1999, 274:2315–2321; Plasminogen activator inhibitor, type II (Planh2) and Planh1, Dougherty K.M., Proc Natl Acad Sci USA 1999, 96:686–691; Protein tyrosine phosphatase, receptor type, B (Ptprb), Elchebly et al. (1999) Science 283:1544–1548; Presenilin 1 (Psen1), Guo Q, et al. (1999) Proc Natl Acad Sci USA, 96:4125–4130; Protein kinase, mitogen-activated 9 (Prkm9) / SAPK/Erk/kinase 2 (Serk2), Kuan CY et al. (1999) Neuron 4:667–676; CD152 antigen (Cd152) / CD86 antigen (Cd86) / CD80 antigen (Cd80), Mandelbrot DA, et al. (1999) J Exp Med, 189:435–440; Poly (ADP-ribose) polymerase (Adprp), Masutani M, et al. (1999), Proc Natl Acad Sci USA 96:2301–2304; Sodium channel, nonvoltage-gated 1 beta (Scnn1b), Pradervand S, et al. (1999) Proc Natl Acad Sci USA 96:1732–1737; Nuclear receptor coactivator 1 (Ncoa1), Qi C, et al. (1999) Proc Natl Acad Sci USA 96:1585–1590; Decay accelerating factor 1 (Daf1), Sun X, et al. (1999) Proc Natl Acad Sci USA 1999, 96:628–633; Necdin (Ndn), Tsai TF, et al. (1999) Nat Genet 22:15–16; Relaxin (Rln); Zhao L, et al. (1999) Endocrinology 140:445–453; Adenylyl cyclase 8 (Adcy8), Abdel-Majid RM, et al. (1998) Nat Genet 19:289–291; Leukemia inhibitory factor (Lif), Bugga L, et al. (1998) J Neurobiol 36:509–524; Lectin, galactose binding, soluble 3 (Lgals3) and Lgals1, Calnot C, et al. (1998) Dev Dyn 211:306–313; Urokinase plasminogen activator receptor (Plaur) Carmeliet P, et al. (1998) J Cell Biol 140:233–245; Nitric oxide synthase 1, neuronal (Nos1), Chao DS, et al. (1998) J Neurochem 71:784–789; Homeo box A7 (Hoxa7), Chen F, et al. (1998) Mech Dev 77:49–57; Myosin light chain, phosphorylatable, cardiac ventricles (Mylpc) Chen J, et al. (1998) J Biol Chem 273:1252–1256; Homeo box B7 (Hoxb7),Chen F, et al. (1998) Mech Dev 77:49–57; Nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, alpha

and beta (Nfkbia and Nfkbib), Cheng JD, et al. (1998) J Exp Med 6:1055–1062; Enolase 1, alpha non-neuron (Eno1), Couldrey C, et al. (1998) Dev Dyn 212:284–292; Xeroderma pigmentosum, complimentation group A (Xpa), De Vries A, et al. (1998) Exp Eye Res 1998, 67:53–59; Von Willebrand factor homolog (Vwf), Denis C, et al. (1998) Proc Natl Acad Sci USA 95:9524–9529; Lysosomal acid lipase 1 (Lip1), Du H, et al. (1998) Hum Mol Genet 7:1347–1354; UNC-5 homolog (C. elegans) 3 (Unc5h3), Eisenman LM, et al. (1998) J Comp Neurol 394:106–117; Protein phosphatase 1, regulatory (inhibitor) subunit 1B (Ppp1r1b), Fienberg AA, et al. (1998) Science 281:838–842; Myelin-associated glycoprotein (Mag) Fujita N, et al. (1998) J Neurosci 18:1970–1978; Paraoxonase 1 (Pon1), Furlong CE, et al. (1998) Neurotoxicology 19:645–650; Brain derived neurotrophic factor (Bdnf), Garek RR, et al. (1998) Laryngoscope 108:671–678; Neurotrophin 3 (Ntf3), Garek RR, et al. (1998) Laryngoscope 168:671–678; Myoglobin (Mb); Garry DJ, et al. (1998) Nature 395:905–908; Opioid receptor, mu (Oprm), Gaveriaux-Ruff C, et al. (1998) Proc Natl Acad Sci USA 95:6326–6330; Neuropeptide Y (Npy), Hollopeter G, et al. (1998) Int J Obes Relat Metab Disord 22:506–512; Procollagen, type I, alpha 1 (Cola1) Hormuzdi SG, et al. (1998) Mol Cell Biol 18:3368–3375; Centromere autoantigen B (Cenpb), Hudson DF, et al. (1998) J Cell Biol 141:309–319; Oculocerebrorenal syndrome of Lowe (ocrl), Janne PA, et al. (1998) J Clin Invest 101:2042–2053; arachidonate 12-lipoxygenase (Alox12) Johnson EN, et al. (1998) Proc Natl Acad Sci USA 95:3100–3105; H19 fetal liver mRNA (H19), Jones BK, et al. (1998) Genes Dev 12:2200–2207; Hepatocyte nuclear factor 3 gamma (winged helix transcription factor) (Hnf3g), Kaestner KH, et al. (1998) Mol Cell Biol 18:4245–4251; Bone morphogenetic protein 2 (Bmp2) / Bone morphogenetic protein 7 (Bmp7) and Bmp5, Katagiri T, et al. (1998) Dev Genet 22:340–348; Intercellular adhesion molecule (Icam1), Ley K, et al. (1998) Circ Res 83:287–294; Glutamyl aminopeptidase (Enpep), Lin Q, et al. J Immunol 1998, 160:4681–4687; Prion protein (Prnp), Lipp HP, et al. Behav Brain Res 1998, 95:47–54; RAB3A, member RAS oncogene family (Rab3a), Lonart G, et al. Neuron 1998, 21:1141–1150; Potassium voltage gated channel, shaker related subfamily, member 4 (Kcna4), London B, et al., J Physiol (Lond) 1998, 509:171–182; Apurinic/apyrimidinic endonuclease (Apex), Ludwig DL, et al. Mutat Res 1998, 409:17–29;

T-cell receptor gamma, variable 5 (Tcrg-V5), Mallick-Wood CA, et al. Science 1998, 279:1729–1733; Nuclear, factor, erythroid derived 2, like 2 (Nfe2l2), Martin F, et al. Blood 1998, 91:3459–3466; Interleukin 13 (Il13), McKenzie GJ, et al. Curr Biol 1998, 8:339–340; Sorbitol dehydrogenase 1 (Sdh1), Ng T, et al. Diabetes 1998, 47:961–966; Guanine nucleotide binding protein, alpha 11 (Gna11), Offermanns S, et al. EMBO J 1998, 17:4304–4312; Estrogen receptor alpha (Estra), Ogawa S, et al. Endocrinology 1998, 139:5070–5081; Integrin beta 2 (Itgb2), Intracellular adhesion molecule (Icam1) and CD34 antigen, Oliveira-dos-Santos AJ, et al. Eur J Immunol 1998, 28:2882–2892; Angiotensin receptor 1b (Agtr1b), Oliverio MI, et al. Proc Natl Acad Sci USA 1998, 95:15496–15501; Complement factor B (factor B), Pekna M, et al. Scand J Immunol 1998, 47:375–380; Centromere autoantigen B (Cenpb), Perez-Castro AV, et al. Dev Biol 1998, 201:135–143; Procollagen, type V, alpha 2 (Col5a2) / Fibrillin 1 (Fbn1), Phelps RG, et al. Mol Med 1998, 4:356–360; Plasminigen activator inhibitor, type 1 (Planh1), Pinsky DJ, et al. J Clin Invest 1998, 102:919–928, Carmeliet P, et al. J Clin Invest 1993, 92:2756–2760; Placentae and embryos oncofetal gene (Pem), Pitman JL, et al. Dev Biol 1998, 202:196–214; Postmeiotic segregation increased 1 (S. cerevisiae) (Pms1), Prolla TA, et al. Nat Genet 1998, 18:276–278; Prion protein, structural locus (Prn-p), Prusiner SB, et al. Proc Natl Acad Sci USA 1998, 90:10608–10612, Lledo P-M, et al. Proc Natl Acad Sci U S A 1996, 93:2403–2407, Sailer A, et al. Cell 1994, 77:967–968, Weissmann C, et al. Philos Trans R Soc Lond [Biol] 1994, 343:431–433, Bueler H, et al. Cell 1993, 73:1337–1347, Weissmann C, et al. Intervirology 1993, 35:164–175; NAD (P)H:quinone oxidoreductase, Radjendirane V, et al. J Biol Chem 1998, 273:7382–7389; Alpha tropomyosin (Tpm1), Rethinasamy P, et al. Circ Res 1998, 82:116–123; Goosecoid and Goosecoid-like (Gscl), Wakamiya M, et al. Hum Mol Genet 1998, 7:1835–1840, Saint-Pore B, et al. Hum Mol Genet 1998, 7:1841–1849; Schlafen 1 (Slfn1); Schwarz DA, et al. Immunity 1998, 9:657–668; Nuclear factor, erythroid derived 2, ubiquitous (Mafk), Shavit JA, et al. Genes Dev 1998, 12:2164–2174; Microphthalmia-associated transcription factor (Mitf), Smith SB, et al. Exp Eye Res 1998, 66:403–410; Bone morphogenetic protein 6 (Bmp6), Solloway MJ, et al. Dev Genet 1998, 22:321–339; Phosphatidylinositol glycan, class A (Piga), Takahama Y, et al. Eur J Immunol 1998,

28:2159–2166; Paired-related homeobox 2 (Prx2), Berge D, et al. Development 1998, 125:3831–3842; Prostaglandin E receptor EP1 subtype (Ptgerep1), Ushikubi F, et al. Nature 1998, 395:281–284; Immunoglobulin kappa chain complex (Igk), van der Stoep N, et al. Immunity 1998, 8:743–750; Adenine phosphoribosyl transferase (Aprt), Van Sloun PP, et al. Nucleic Acids Res 1998, 26:4888–4894; Microtubule associated protein 4 (Mtap4), Voss AK, et al. Dev Dyn 1998, 212:258–266; ; 3-hydroxy-3-methylglutaryl-coenzyme A lyase (Hmgcl), Wang SP, et al. Hum Mol Genet 1998, 7:2057–2062; Fibroblast growth factor receptor 4 (Fgfr4), Weinstein M, et al. Development 1998, 125:3615–3623; Hepsin (Hpn), Wu Q, et al. J Clin Invest 1998, 101:321–326; Small inducible cytokine A11 (Scya11), Yang T, et al. Blood 1998, 92:3912–3923; Small nuclear ribonucleoprotein N (Snrpn), Yang T, et al. Nat Genet 1998, 19:25–31; DNA fragmentation factor, alpha subunit (Dffa), Zhang J, et al. Proc Natl Acad Sci USA 1998, 95:12480–12485; Early growth response 1 (Egr1), Zheng D, et al. Neuroscience 1998, 83:251–258; Early growth response 1 (Egr1) / Hormone receptor (Hmr), Zheng D, et al. Neuroscience 1998, 83:251–258; Hemochromatosis (Hfe),Zhou XY, et al. Proc Natl Acad Sci USA 1998, 95:2492–2497; Alpha tropomyosin (Tpm1), Blanchard EM, et al. Circ Res 1997, 81:1005–1010; tRNA phosphoserine (Trsp), Bosl MR, et al. Proc Natl Acad Sci U S A 1997, 94:5531–5534; Angiotensin receptor 1b (Agtr1b), Chen X, et al. Am J Physiol 1997, 272:F299–F304; Xeroderma pigmentosum, complementation group C (Xpc), Cheo DL, et al. Mut Res 1997, 374:1–9; B cell leukemia/lymphoma 6 (Bcl6), Dent AL, et al. Science 1997, 276:589–592; Fumarylacetoacetate hydrolase (Fah) / 4-hydroxyphenylpyruvic acid dioxygenase (Hpd), Endo F, et al. J Biol Chem 1997, 272:24426–24432; N-methylpurine-DNA glycosylase (Mpg), Engelward BP, et al. Proc Natl Acad Sci USA 1997, 94:13087–13092; Interleukin 1 receptor, type 1 (Il1r1), Glaccum MB, et al. J Immunol 1997, 159:3364–3371; N-methylpurine-DNA glycosylase (Mpg), Hang B, et al.Proc Natl Acad Sci USA 1997, 94:12869–12874; Gamma-aminobutyric acid (GABA-A) receptor, subunit alpha 6 (Gabra6), Homanics GE, et al. Mol Pharmacol 1997, 51:588–596; Superoxide dismutase 2, mitochondrial (Sod2), Huang TT, et al. Arch Biochem Biophys 1997, 344:424–432; Interleukin 11 receptor, alpha chain 1 (Il11ra1), Nandurjar HH, et al. Blood 1997, 90:2148–

2159; Alkaline phosphatase 5 (Akp5), Narisawa S, et al. Dev Dyn 1997, 208:432–446; GATA-binding protein 4 (Gata4), Narita N, et al. Development 1997, 124:3755–3764; Lymphocyte protein tyrosine kinase (Lck) / Fyn protooncogene (Fyn); Page ST, et al. Eur J Immunol 1997, 27:554–562; P glycoprotein 3 (Pgy3), Schinkel AH, et al. Proc Natl Acad Sci U S A 1997, 94:4028–4033; P glycoprotein 1 (Pgy1) / P glycoprotein 3 (Pgy3), Schinkel AH, et al., Proc Natl Acad Sci U S A 1997, 94:4028–4033; Creatine kinase, mitochondrial 1, ubiquitous (Ckmt1), Steeghs K, et al. J Neurosci Methods 1997, 71:29–41; T cell receptor gamma, variable 4 (Tcrg-V4), Sunaga S, et al. J Immunol 1997, 158:4223–4228; Ia-associated invariant chain (p31 form) (Ii), Takaesu NT, et al. J Immunol 1997, 158:187–199; Solute carrier family 18 (vesicular monoamine), member 2 (Slc18a2), Takahashi N, et al. Proc Natl Acad Sci USA 1997, 94:9938–9943; Matrix metalloproteinase 7 (Mmp7), Wilson CL, et al. Proc Natl Acad Sci U S A 1997, 94:1402–1407; Formin (Fmn),Wynshaw-Boris A, et al. Mol Med 1997, 3:372–384; Sypnaptophysin (Syp), Arrandale JM, et al. J Biol Chem 1996, 271:21353–21358; Transformation related protein 53 (Trp53), Boehme SA, et al. J Immunol 1996, 156:4075–4078; Neuronal nitric oxide synthase (Nos1), Burnett AL, et al. Mol Medicine 1996, 2:288–296; Eph receptor A2 (Epha2), Chen J, et al. Oncogene 1996, 12:979–988; Urokinase plasminogen activator receptor (Plaur); Dewerchin M, et al. J Clin Invest 1996, 97:870–878; Growth differentiation factor 9 (Gdf9), Dong J, et al. Nature 1996, 383:531–535; Externally regulated phosphatase (Ptpn16), Dorfman K, et al. Oncogene 1996, 13:925–931; Tenascin C (Tnc), Forsberg E, et al. Proc Natl Acad Sci U S A 1996, 93:6594–6599; Integrin alpha 1 (Itga1), Gardner H, et al. Dev Biol 1996, 175:301–313; FBJ osteosarcoma oncogene B (Fosb), Gruda MC, et al. Oncogene 1996, 12:2177–2185; Breast cancer 1 (Brca1), Hakem R, et al. Cell 1996, 85:1009–1023; Megakaryocyte-associated tyrosine kinase (Matk), Hamaguchi I, et al. Biochem Biophys Res Commun 1996, 224:172–179; Apolipoprotein B editing complex 1 (Apobec1), Hirano K-I, et al. J Biol Chem 1996, 271:9887–9890; Carboxyl ester lipase, Howles PN, et al. J. Biol. Chem. 1996, 271:7196–7202; Nuclear factor, erythroid derived 2, ubiquitous (Nfe2u), Kotkow KJ, Orkin SH. Proc Natl Acad Sci U S A 1996, 93:3514–3518; Retinoid X receptor gamma (Rxrg), Krezel W, et al. Proc Natl Acad Sci U S A 1996, 93:9010–9014; Early growth response 1 (Egr1), Lee SL,

et al. Mol Cell Biol 1996, 16:4566–4572; Adrenergic receptor, alpha 2b (Adra2b), Link RE, et al. Science 1996, 273:803–805; Angiotensin receptor 1a (Agtr1a), Matsusaka T, et al. J Clini Invest 1996, 98:1867–1877; Interleukin 3 receptor, beta chain 1 (Il3rb2), Nicola NA, et al. Blood 1996, 87:2665–2674; Transformation related protein 53 (Trp53), Ohashi M, et al. Jpn J Cancer Res 1996, 87:696–701; Leukemia-associated gene (Lag), Schubart U K, et al. J Biol Chem 1996, 271:14062–14066; Glutathione peroxidase 1 (Gpx1), Spector A, et al. Exp Eye Res 1996, 62:521–540; Arachidonate 12-lipoxygenase, leukocyte (Alox12l), Sun D, Funk CD. J Biol Chem 1996, 271:24055–24062; Complement component 3 (C3), Sylvestre D, et al. J Exp Med 1996, 184:2385–2392; CD30 antigen (Cd30), Texido G, et al. Eur J Immunol 1996, 26:1966–1969; Fc receptor, IgE, low affinity II, alpha polypeptide (Fcer2a), Immunoglobulin heavy chain 5 (delta-like heavy chain) (Igh-5), Interleukin 4 (IL4) and Terminal-deoxynucleotidyl transferase, Texido G, et al. Eur J Immunol 1996, 26:1966–1969; Apolipoprotein A-II (Apoa2), Weng W, Breslow JL. Proc Natl Acad Sci U S A 1996, 93:14788–14794; Amyloid beta (A4) precursor protein (App), Zheng H, et al. Ann N Y Acad Sci 1996, 777:421–426; cAMP responsive element binding protein 1 (Creb1), Blendy JA, et al. Brain Res 1995, 681:8–14; Bradykinin receptor, beta 2 (Bdkrb2), Borkowski JA, et al. J Biol Chem 1995, 270:13706–13710; Growth factor response protein (Gfrp), Crawford PA, et al. Mol Cell Biol 1995, 15:4331–4336; Ciliary neurotrophic factor (Cntf), de Chiara TM, et al. Cell 1995, 83:313–322; Cyclin dependent kinase inhibitor 1A (P21) (Cdkn1a); Deng C, et al. Cell 1995, 82:675–684; Granzyme A (Gzma), Ebnet K, et al. EMBO J 1995, 14:4230–4239; Very low density lipoprotein receptor (Vldlr), Frykman PK, et al. Proc Natl Acad Sci U S A 1995, 92:8453–8457; Apolipoprotein E (Apoe) / Apolipoprotein A-I (Apoa1), Goodrum JF, et al. J Neurobiol 1995, 64:408–416; Nitric oxide synthase 1, neuronal (Nos1), Ichinose F, et al. Anesthesiology 1995, 83:101–108; Nitric oxide synthase 2, inducible, macrophage (Nos2), Laubach VE, et al. Proc Natl Acad Sci U S A 1995, 92:10688–10692; Peroxisome proliferator activated receptor alpha (Ppara), Lee SS-T, et al. Mol Cell Biol 1995, 15:3012–3022; Growth factor response protein (Gfrp), Lee SL, et al. Science 1995, 269:532–535; H19 fetal liver mRNA (H19) / Insulin-like growth factor 2 (Igf2), Leighton PA, et al. Nature 1995, 375:34–39; Retinoic acid receptor beta (RAR-beta),

Luo J, et al. Mech Dev 1995, 53:61–71; Metallothionein 1 (Mt1) / Metallothionein 2 (Mt2), Philcox JC, et al. Biochem J 1995, 308:543–546; Heme oxygenase (decycling) 2 (Hmox2), Poss KD, et al. Neuron 1995, 15:867–873; H1-0 histone (H1fv), Sirotkin AM, et al. Proc Natl Acad Sci U S A 1995, 92:6434–6438; Creatine kinase, mitochondrial 1, ubiquitous (Ckmt1), Steeghs K, et al. Biochim Biophys Acta 1995, 1230:130–138; Tenascin C (Tnc), Steindler DA, et al. J Neurosci 1995, 15:1971–1983; Ia-associated invariant chain (Ii), Takaesu NT, et al. Immunity 1995, 3:385–396; Neuroblastoma ras oncogene (Nras), Umanoff H, et al. Proc Natl Acad Sci USA 1995, 92:1709–1713; Receptor-associated protein of the synapse, 43 kDa (Rapsn), Willnow TE, et al. Proc Natl Acad Sci USA 1995, 92:4537–4541; Vitronectin (Vtn), Zheng X, et al. Proc Natl Acad Sci U S A 1995, 92:12426–12430; Preproacrosin (Acr), Baba T, et al. J Biol Chem 1994, 269:31845–31849; Vimentin (Vim), Colucci GE, et al. Cell 1994, 79:679–694; Tumor necrosis factor receptor superfamily, member 1b (Tnfrsf1b), Erickson SL, et al. Nature 1994, 372:560–563; Cellular retinoic acid binding protein I (Crabp1), Gorry P, et al. Proc Natl Acad Sci USA 1994, 91:9032–9036; cAMP responsive element binding protein 1 (Creb1), Hummler F, et al. Proc Natl Acad Sci USA 1994, 91:5647–5651; Pore forming protein (Pfp), Kagi D, et al. Nature 1994, 369:31–37; Src-related kinase lacking C-terminal regulatory tyrosine and N-terminal myristylation sit (Srms), Kohmura N, et al. Mol Cell Biol 1994, 14:6915–6925; CD3 polypeptide zeta (Cd3z) / Solute carrier family 22, member 1 (Slc22a1), Koyasu S, et al. EMBO J 1994, 13:784–797; Pore forming protein (Pfp), Lowin B, et al. Proc Natl Acad Sci USA 1994, 91:11571–11575; Retinoic acid receptor, beta (Rarb), Retinoic acid receptor beta2 (RARbeta2), Mendelsohn C, et al. Dev Biol 1994, 166:246–258; Transthyretin (Ttr), Palha JA, et al. J Biol Chem 1994, 269:33135–33139; Procollagen, type X, alpha 1 (Col10a1), Rosati R, et al. Nature Genet 1994, 8:129–135; P glycoprotein 3 (Pgy3), Schinkel AH, et al. Cell 1994, 77:491–502; Yamaguchi sarcoma viral (v-yes) oncogene homolog (Yes), Stein PL, et al. Genes Dev 1994, 8:1999–2007; Fc receptor, IgE, high affinity II, alpha polypeptide (Fcer2a), Stief A, et al. J Immunol 1994, 152:3378–3390; Pore forming protein (Pfp), Walsh CM, et al. Proc Natl Acad Sci USA 1994, 91:10854–10858; CD2 antigen (Cd2), Evans CF, et al. J Immunol 1993, 151:6259–6264; Mannose-6-

phosphate receptor, cation dependent (M6pr), Koster A, et al. EMBO J 1993, 12:5219–5223; Retinoic acid receptor, alpha (Rara), Li E, et al. Proc Natl Acad Sci USA 1993, 90:1590–1594, Lufkin T, et al. Proc Natl Acad Sci USA 1993, 90:7225–7229; Retinoic acid receptor, gamma (Rarg), Lohnes D, et al. Cell 1993, 73:643–658; Tumor necrosis factor receptor 1 (TNF-R-1) (Tnfr1), Pfeffer K, et al. Cell 1993, 73:457–467; Lectin, galactose binding, soluble 1 (Lgals1), Poirier F, Robertson EJ. Development 1993, 119:1229–1236; Synapsin I (Syn1), Rosahl TW, et al. Cell 1993, 75:661–670; Tumor necrosis factor receptor 1 (Tnfr1), Rothe J, et al. Nature 1993, 364:798–802; Beta-2 microglobulin (B2m), Correa I, et al. Proc Natl Acad Sci USA 1992, 89:653–657; CD2 antigen (Cd2), Killeen N, et al. EMBO J 1992, 11:4329–4336; Apolipoprotein E (Apoe), Piedrahita JA, et al. Proc Natl Acad Sci USA 1992, 89:4471–4475; Myogenic differentiation 1 (Myod1), Rudnicki MA, et al. Cell 1992, 71:383–390; Tenascin C (Tnc), Saga Y, et al. Genes Dev 1992, 6:1821–1831; Beta-2 microglobulin (B2m), Sanjuan N, et al. J Virol 1992, 66:4587–4590; Neuroblastoma myc-related oncogene 1 (Nmyc1), Stanton BR, et al. Genes Dev 1992, 6:2235–2247; and Hemoglobin alpha.chain complex (Hba), Popp RA, et al. Genetics 1983, 105:157–167.

Some preferred single-copy, non-essential genes with no phenotypes of the present invention include, but are not limited to, the following: Moesin (Msn), Doi Y., et al., J Biol Chem 1999, 274:2315–2321; Plasminogen activator inhibitor, type II (Planh2) and Planh1; Dougherty K.M., Proc Natl Acad Sci USA 1999, 96:686–691; Nuclear receptor coactivator 1 (Ncoa1), Qi C, et al. (1999) Proc Natl Acad Sci USA 96:1585–1590; Nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, alpha and beta (Nfkbia and Nfkbib), Cheng JD, et al. (1998) J Exp Med 6:1055–1062; H19 fetal liver mRNA (H19), Jones BK, et al. (1998) Genes Dev 12:2200–2207; Prion protein (Prnp), Lipp HP, et al. Behav Brain Res 1998, 95:47–54; Centromere autoantigen B (Cenpb), Perez-Castro AV, et al. Dev Biol 1998, 201:135–143; Placentae and embryos oncofetal gene (Pem), Pitman JL, et al. Dev Biol 1998, 202:196–214; Externally regulated phosphatase (Ptpn16), Dorfman K, et al. Oncogene 1996, 13:925–931; Transformation related protein 53 (Trp53), Ohashi M, et al. Jpn J Cancer Res 1996, 87:696–701; H1-0 histone (H1fv), Sirotkin AM, et al. Proc Natl Acad Sci U S A 1995, 92:6434–6438; Creatine kinase, mitochondrial 1, ubiquitous (Ckmt1), Steeghs K, et

al. Biochim Biophys Acta 1995, 1230:130–138; Neuroblastoma ras oncogene (Nras), Umanoff H, et al. Proc Natl Acad Sci USA 1995, 92:1709–1713; Vitronectin (Vtn), Zheng X, et al. Proc Natl Acad Sci U S A 1995, 92:12426–12430; Vimentin (Vim), Colucci GE, et al. Cell 1994, 79:679–694; Cellular retinoic acid binding protein I (Crabp1), Gorry P, et al. Proc Natl Acad Sci USA 1994, 91:9032–9036; Retinoic acid receptor beta2 (RARbeta2), Mendelsohn C, et al. Dev Biol 1994, 166:246–258; Retinoic acid receptor, alpha (Rara), Li E, et al. Proc Natl Acad Sci USA 1993, 90:1590–1594, Lufkin T, et al. Proc Natl Acad Sci USA 1993, 90:7225–7229; Lectin, galactose binding, soluble 1 (Lgals1), Poirier F, Robertson EJ. Development 1993, 119:1229–1236; Myogenic differentiation 1 (Myod1), Rudnicki MA, et al. Cell 1992, 71:383–390; and Tenascin C (Tnc), Saga Y, et al. Genes Dev 1992, 6:1821–1831.

In view of the guidance of the present specification, one of ordinary skill in the art can select similar, suitable, single-copy, non-essential genes in mice and other cell types/organisms.

Assembly of Targeting Cassettes

The targeting cassettes described herein can be constructed utilizing methodologies known in the art of molecular biology (see, for example, Ausubel or Maniatis) in view of the teachings of the specification. As described above, the targeting constructs are assembled by inserting, into a suitable vector backbone, polynuclotides encoding a reporter, such as a light-generating protein, *e.g.,* a luciferase gene, operably linked to a promoter of interest; a sequence encoding a positive selection marker; and, optionally a sequence encoding a negative selection marker. In addition, the targeting cassette contains insertion sites such that sequences targeting a single-copy, non-essential gene can be readily inserted to flank the sequence encoding positive selection marker and luciferase-encoding sequence.

A preferred method of obtaining polynucleotides, suitable regulatory sequences (*e.g.,* promoters) is PCR. General procedures for PCR as taught in MacPherson et al., PCR: A PRACTICAL APPROACH, (IRL Press at Oxford University Press, (1991)). PCR conditions for each application reaction may be empirically determined. A number of parameters influence

the success of a reaction. Among these parameters are annealing temperature and time, extension time, Mg2+ and ATP concentration, pH, and the relative concentration of primers, templates and deoxyribonucleotides. Exemplary primers are described below in the Examples. After amplification, the resulting fragments can be detected by agarose gel electrophoresis followed by visualization with ethidium bromide staining and ultraviolet illumination.

In one embodiment, PCR can be used to amplify fragments from genomic libraries. Many genomic libraries are commercially available. Alternatively, libraries can be produced by any method known in the art. Preferably, the organism(s) from which the DNA is has no discernible disease or phenotypic effects. This isolated DNA may be obtained from any cell source or body fluid (*e.g.*, ES cells, liver, kidney, blood cells, buccal cells, cerviovaginal cells, epithelial cells from urine, fetal cells, or any cells present in tissue obtained by biopsy, urine, blood, cerebrospinal fluid (CSF), and tissue exudates at the site of infection or inflammation). DNA is extracted from the cells or body fluid using known methods of cell lysis and DNA purification. The purified DNA is then introduced into a suitable expression system, for example a lambda phage.

Another method for obtaining polynucleotides, for example, short, random nucleotide sequences, is by enzymatic digestion. As described below in the Examples, short DNA sequences generated by digestion of DNA from vectors carrying genes encoding luciferase (yellow green or red).

Polynucleotides are inserted into vector genomes using methods known in the art. For example, insert and vector DNA can be contacted, under suitable conditions, with a restriction enzyme to create complementary or blunt ends on each molecule that can pair with each other and be joined with a ligase. Alternatively, synthetic nucleic acid linkers can be ligated to the termini of a polynucleotide. These synthetic linkers can contain nucleic acid sequences that correspond to a particular restriction site in the vector DNA. Other means are known and, in view of the teachings herein, can be used.

The final constructs can be used immediately (*e.g.,* for introduction into ES cells), or stored frozen (*e.g.,* at -20°C) until use. Preferably, the constructs are linearized prior to use, for example by digestion with suitable restriction endonucleases.

Transgenic Animals

The targeting constructs containing the luciferase genes are introduced into a pluripotent cell (*e.g.,* ES cell, Robertson, E. J., In: Current Communications in Molecular Biology, Capecchi, M. R. (ed.), Cold Spring Harbor Press, Cold Spring Harbor, N.Y. (1989), pp. 39-44). Suitable ES cells may be derived or isolated from any species or from any strain of a particular species. Although not required, the pluripotent cells are typically derived from the same species as the intended reciepient. ES cells may be obtained from commercial sources, from International Depositories (*e.g.,* the ATCC) or, alternatively, may be obtained as described in Robertson, E. J., *supra.* Examples of clonally-derived ES cells lines include 129/SVJ ES cells, RW-4 and C57BL/6 ES cells (Genome Systems, Inc.).

ES cells are cultured under suitable conditions, for example, as described in Ausubel et al., section 9.16, *supra.* Preferably, ES cells are cultured on stomal cells (such as STO cells (especially SNC4 STO cells) and/or primary embryonic fibroblast cells) as described by E. J. Robertson, *supra,* pp 71-112. Culture media preferably includes leukocyte inhibitory factor ("lif") (Gough, N. M. et al., Reprod. Fertil. Dev. 1:281-288 (1989); Yamamori, Y. et al., Science 246:1412-1416 (1989), which appears to help keep the ES cells from differentiating in culture. Stomal cells transformed with the gene encoding lif can also be used.

The targeting constructs are introduced into the ES cells by any method which will permit the introduced molecule to undergo recombination at its regions of homology, for example, micro-injection, calcium phosphate transformation, or electroporation (Toneguzzo, F. et al., Nucleic Acids Res. 16:5515-5532 (1988); Quillet, A. et al., J. Immunol. 141:17-20 (1988); Machy, P. et al., Proc. Natl. Acad, Sci. (U.S.A.) 85:8027-8031 (1988)). The construct to be inserted into the ES cell must first be in the linear form. Thus, if the knockout construct has been inserted into a vector as described above, linearization is

accomplished by digesting the DNA with a suitable restriction endonuclease selected to cut only within the vector sequence and not within the knockout construct sequence. If the ES cells are to be electroporated to insert the construct, the ES cells and construct DNA are exposed to an electric pulse using an electroporation machine and following the manufacturer's guidelines for use. After electroporation, the ES cells are typically allowed to recover under suitable incubation conditions. The cells are then cultured under conventional conditions, as are known in the art, and screened for the presence of the construct.

Screening and selection of those cells into which the targeting construct has been integrated can be achieved using the positive selection marker and/or the negative selection marker in the construct. In preferred embodiments, the construct contains both positive and negative selection markers. In one aspect, methods which rely on expression of the selection marker are used, for example, by adding the appropriate substrate to select only those cells which express the product of the positive selection marker or to eliminate those cells expressing the negative selection marker. For example, where the positive selection marker encodes neomycin resistance, G418 is added to the transformed ES cell culture media at increasing dosages. Similarly, where the negative selection marker is used, a suitable substrate (*e.g.,* gancyclovir if the negative selection marker encodes HSV-TK) is added to the cell culture. Either before or after selection using the appropriate substrate, the presence of the positive and/or negative selection markers in a recipient cell can also be determined by others methods, for example, hybridization, detection of radiolabelled nucleotides, PCR and the like. In preferred embodiments, cells having integrated targeting constructs are first selected by adding the appropriate substrate for the positive and/or negative selection markers. Cells that survive the selection process are then screened by other methods, such as PCR or Southern blotting, for the presence of integrated sequences.

After suitable ES cells containing the construct in the proper location have been identified, the cells can be inserted into an embryo, preferably a blastocyst. The blastocyts are obtained by perfusing the uterus of pregnant females. In one embodiment, the blastocyts are obtained from, for example, the FVB/N strain of mice and the ES cells are obtained from, for example, the C57BL/6 strain of mice. Suitable methods for accomplishing this are

known to the skilled artisan, and are set forth by, e.g., Bradley et al., (1992) Biotechnology, 10:534-539. Insertion into the embryo may be accomplished in a variety of ways known to the skilled artisan, however a preferred method is by microinjection. For microinjection, about 10-30 ES cells are collected into a micropipet and injected into embryos that are at the proper stage of development to permit integration of the foreign ES cell containing the construct into the developing embryo. The suitable stage of development for the embryo used for insertion of ES cells is species dependent, in mice it is about 3.5 days.

While any embryo of the right stage of development is suitable for use, it is preferred that blastocysts are used. In addition, preferred blastocysts are male and, furthermore, preferably have genes encoding a coat color that is different from that encoded by the genes ES cells. In this way, the offspring can be screened easily for the presence of the knockout construct by looking for mosaic coat color (indicating that the ES cell was incorporated into the developing embryo). Thus, for example, if the ES cell line carries the genes for black fur, the blastocyst selected will carry genes for white or brown fur.

After the ES cell has been introduced into the blastocyst, the blastocyst is typically implanted into the uterus of a pseudopregnant foster mother for gestation. Pseudopregnant females are prepared by mating with vasectomized males of the same species and successful implantation usually must occur within about 2-3 days of mating.

Offspring are screened initially for mosaic coat color where the coat color selection strategy has been employed. Southern blots and/or PCR may also be used to determine the presence of the sequences of interest. Mosaic (chimeric) offspring are then bred to each other to generate homozygous animals. Homozygotes and heterozygotes may be identified by Southern blotting of equivalent amounts of genomic DNA from mice that are the product of this cross, as well as mice that are known heterozygotes and wild type mice. Alternatively, Northern blots can be used to probe the mRNA to identify the presence or absence of transcripts encoding either the replaced gene, the luciferase gene, or both. In addition, Western blots can be used to assess the level of expression of the luciferase protein with an antibody against the luciferase gene product. Finally, in situ analysis (such as fixing the cells and labeling with antibody) and/or FACS (fluorescence activated cell sorting)

analysis of various cells from the offspring can be conducted using suitable (*e.g.*, anti-luciferase) antibodies to look for the presence or absence of the targeting construct.

In one embodiment of the present invention, the animals are from the C57BL/6 mouse strain. This strain develops a variety of tumors and has been used to develop a number of tumor cells lines, for example, B16 melanoma cells (including, B16F10, B16D5, and B16F1), Lewis lung carcinoma cells (including, LLC, LLC-h59), T241 mouse fibrosarcoma cells, RM-1 and pTC2 mouse prostate cancer cells, and MCA207 mouse sarcoma cells. These cell lines have been extensively used for *in vivo* tumor biology studies after injection into C57BL/6 mice. The generated targeted transgenic mice in the Examples are in C57BL/6 genetic background and these animals are suitable for injection or implantation of such tumor cells, as well as other tumor cells described in literature that are immunocompatent for C57BL/6 mice. Thus, the transgenic animals can then be used, for example, to monitor, *in vivo*, tumor progression (*e.g.*, growth) and the efficacy of therapies on tumor regression. For example, where the transgenic animal is tumor-susceptible, it is monitored for expression of a reporter, *e.g.*, luciferase, which is indicative of tumorigenesis and/or angiogenesis. The monitoring of expression of luciferase reporter expression cassettes using non-invasive whole animal imaging has been described (Contag, C. et al, U.S. Patent No. 5,650,135, July 22, 1997, herein incorporated by reference; Contag, P., et al, *Nature Medicine* **4**(2):245-247, 1998; Contag, C., et al, *OSA TOPS on Biomedical Optical Spectroscopy and Diagnostics* **3**:220-224, 1996; Contag, C.H., et al, *Photochemistry and Photobiology* **66**(4):523-531, 1997; Contag, C.H., et al, *Molecular Microbiology* **18**(4):593-603, 1995). Such imaging typically uses at least one photo detector device element, for example, a charge-coupled device (CCD) camera.

The transgenic animals described herein can also be used to determine the effect of an analyte (*e.g.*, therapy), for example on tumor progression where the promoter induces luciferase expression when a tumor develops. Methods of administration of the analyte include, but are not limited to, injection (subcutaneously, epidermally, intradermally), intramucosal (such as nasal, rectal and vaginal), intraperitoneal, intravenous, oral or intramuscular. Other modes of administration include oral and pulmonary administration,

suppositories, and transdermal applications. Dosage treatment may be a single dose schedule or a multiple dose schedule. For example, the analyte of interest can be administered over a range of concentration to determine a dose/response curve. The analyte may be administered to a series of test animals or to a single test animal (given that response to the analyte can be cleared from the transgenic animal).

VEGF and VEGFR Genes

In one aspect the present invention relates to the isolation and characterization of the mouse VEGFR-2 gene promoter. This section describes some information related to the VEFG and VEGFR gene families. Alternative names for some of these genes are as follows: VEGF (vascular endothelial growth factor)is also named VPF (vascular permeability factor); VEGFR-1 is also named FLT1; VEGFR-2 is also named KDR/FLK1; and VEGFR-3 is also named FLT4.

VEGF is a homodimeric 45 kDa (monomer 23 kDa) protein. VEGF has five isoforms of which VEGF165 and VEGF121 are the most abundant. Both are ligands for VEGFR-2 as well as VEGFR-1 (Soker, S., et al., JBC 271:5761-67, 1996). VEGF165 is the only VEGF isoform that binds to Neuropillin-1 (Soker, S., et al., Cell 92:735-745, 1998). VEGF is extremely unstable -- its half life in circulation is only 3 minutes (Ferrara, N., et al., Nature 380:439-442, 1996; Ferrara, N., et al., Endocr Rev 18:4-25, 1997).

VEGF-B is 43% (aa) identical to VEGF and exists as homodimers. It can also form heterodimers with VEGF (Olofsson, B., et al., Proc Natl Acad Sci USA 93:2576-81, 1996). VEGF-B is a ligand for VEGFR-1 (Olofsson, B., et al., Proc Natl Acad Sci USA 95:11709-14, 1998).

VEGF-C is 30% (aa) identical to VEGF. The mature VEGF-C is 23 kDa, the precursor protein is 35.8 kDa. VEGF-C is a ligand for VEGFR-3 as well as VEGFR-2. It induces autophosphorylation of both receptors (Joukov, V., et al., EMBO J 15:290-298, 1996).

VEGF-D is 31% (aa) identical to VEGF165 and 48% (aa) identical to VEGF-B. The mature VEGF-D is approximately 22 KDa. VEGF-D is a ligand for VEGFR-3 as well as

VEGFR-2. It induces autophosphorylation of both receptors (Achen, M.G., et al., 1998 Proc Natl Acad Sci USA 95:548-53, 1998).

PIGF is 46% identical (aa) to VEGF (Maglione, D., et al., Proc Natl Acad Sci 88:9267-71, 1991) and can form heterodimers with VEGF ((Disalvo, J., et al., JBC 270:7717-23, 1995).

VEGFR-1 is an approximately 180 KDa tyrosine kinase receptor for VEGF-B (Olofsson, B., et al., Proc Natl Acad Sci USA 95:11709-14, 1998) and VEGF (de Vries, C., et al., Science 255:989-91, 1992) and PIGF (Park, J.E., et al., J Biol Chem 269:25646-54, 1994).

VEGFR-2 is an approximately 200 KDa tyrosine kinase receptor for VEGF (Terman, B.I., et al., Oncogene Sept 6(9):1677-83, 1991), VEGF-C (Joukov, V., et al., EMBO J 15:290-298, 1996), and VEGF-D (Achen, M.G., et al., 1998 Proc Natl Acad Sci USA 95:548-53, 1998).

VEGFR-3 is a tyrosine kinase receptor (Pajusola, K., et al., Cancer Res 52:5738-43, 1992) on lymphatic EC for VEGF-C (Dumont, D.J., et al., Science 282:946-949, 1998) and VEGF-D (Achen, M.G., et al., 1998 Proc Natl Acad Sci USA 95:548-53, 1998). VEGFR-3 has a processed mature form of about 125 kDa, and an unprocessed form of about 195 kDa (Achen, M.G., et al., 1998 Proc Natl Acad Sci USA 95:548-53, 1998).

Neuropillin-1 is an approximately 130 KDa receptor tyrosine kinase. It binds VEGF165, but not VEGF121 (Soker, S., et al., Cell 92:735-745, 1998).

Expression of many of these genes has been evaluated in adults. A summary of information relating to expression follows here.

VEGF has an approximately 3.7 kb transcript. It is expressed in multiple human tissues, including heart, skeletal muscle and prostate. In mouse, VEGF is mainly expressed in heart, lung and kidney. The rest of the human or mouse tissues, including brain and testis, do not express detectable or significant level of VEGF (Olofsson, B., et al., Proc Natl Acad Sci USA 93:2576-81, 1996). In another study, it was shown that VEGF is highly expressed in epithelial cells of lung alveoli, renal glomeruli and adrenal cortex and in cardiac myocytes (Berse, B., MCB 3:211-20, 1992).

VEGF-B has an approximately 1.4 kb transcript. It is expressed in a majority of human and mouse tissues. In human, VEGF-B is most prominently expressed in heart, skeletal muscle, pancreas, brain and prostate. In mouse, VEGF-B is mostly expressed heart, skeletal muscle, brain and kidney. Liver does not appear to express a significant level of VEGF-B in either humans or mice. VEGF-B and VEGF are co-expressed in many human tissues, such as heart, skeletal muscle, pancreas and prostate. In general, VEGF-B is more abundantly expressed than VEGF. VEGF-B can act as an endothelial cell growth factor (Olofsson, B., et al., Proc Natl Acad Sci USA 93:2576-81, 1996).

VEGF-C has an approximately 2.4 kb transcript that is expressed in multiple human tissues, most prominently in heart, skeletal muscle, placenta, ovary, small intestine, pancreas and prostate. Several tissues, including brain and liver, do not appear to express detectable levels of VEGF-C (Joukov, V., et al., EMBO J 15:290-298, 1996).

VEGF-D has an approximately 2.3 kb transcript that is expressed in multiple human tissues, most prominently in heart, skeletal muscle, lung, colon and small intestine. Several tissues, including brain, liver, placenta, do not appear to express detectable levels of VEGF-D (Achen, M.G., et al., 1998 Proc Natl Acad Sci USA 95:548-53, 1998).

VEGFR-1 appears to be endothelial cell specific (Peters, K.G., et al., Proc Natl Acad Sci 90:8915-19, 1993). VEGFR-1 cDNA is approximately 7.7 kb and encodes a protein of 1338 aa. It was expressed in a variety of normal tissues of adult rat (Shibuya, M., et al., Oncogene 5:519-24, 1990). In a glioma model of tumor angiogenesis, both VEGFR-1 and VEGFR-2 are specifically expressed in Ecs that have penetrated into the tumor, but are absent from Ecs in the normal brain tissues. VEGF expression was detectable in glioma cells along necrotic edge (Plate, K.H., et al., Cancer Research 53:5822-27, 1993).

VEGFR-2 is expressed as an approximately 7 kb transcript (Terman, B.I., et al., Oncogene Sept 6(9):1677-83, 1991) that appears to be endothelial cell specific. VEGFR-2 is expressed ubiquitously in many tissues, including heart, placenta, lung and kidney. The expression levels of VEGFR-2 are relatively low in these tissues compared with neuropillin expression. Brain does not appear to express detectable levels of VEGFR-2 (Soker, S., et al., Cell 92:735-745, 1998). *In situ* hybridization analysis revealed a specific association of

VEGFR-2 with endothelial cells at all stages of mouse development. It is abundant in proliferating endothelial cells of vascular sprouts and branching vessels of embryonic and early postnatal brain, but were drastically reduced in adult brain, where proliferation has ceased (Millauer, B., Cell 72:835-46, 1993).

VEGFR-3 is expressed as approximately 5.8 kb and 4.5 kb mRNAs. Most fetal tissues expressed VEGFR-3, with spleen, brain intermediate zone, and lung showing the highest levels. It does not appear to be expressed in the endothelial cells of blood vessels (Pajusola, K., et al., Cancer Res 52:5738-43, 1992). During embryonic development, VEGFR-3 is expressed in blood vessels but become largely restricted to the lymphatic endothelium postnatally (Kaipainen, A., et al., Proc Natl Acad Sci USA 92: 3566-3570, 1995).

Neuropillin-1 is expressed in both endothelial cells and many types of tumor cells as an approximately 7 kb transcript. Most tissues express high level of Neuropillin-1, especially in heart and placenta. Skeletal muscle, pancreas, lung and kidney also express high level of Neuropillin-1. Brain does not appear to express detectable levels of Neuropillin-1 (Soker, S., et al., Cell 92:735-745, 1998).

Some functions of these genes have been evaluated and are as follows.

VEGF is a specific mitogen for EC *in vitro* and a potent angiogenic factor *in vivo*. *In vitro*, VEGF binds and induces autophosphorylation of VEGFR-2 and VEGFR-1, but the mitogenic response is mediated only through VEGFR-2 (Waltenberger, J., JBC 269:26988-95, 1994). VEGF functions as a survival factor for newly formed vessels during developmental neovascularization, possibly through mediating interaction of endothelial cells with underlying matrix, but is not required for maintenance of mature vessels (Benjamin, L.E., et al., Proc Natl Acad Sci 94:8761-66, 1997). In embryogenesis, VEGF and VEGFR-2 interaction induces the birth and proliferation of endothelials (Hanahan, D., Science 277:48-50, 1997). Binding of VEGF to VEGFR-1 elicits endothelial cell-cell interactions and capillary tube formation, a process that follows closely proliferation and migration of endothelial cells (Hanahan, D., Science 277:48-50, 1997). In a tumorigenesis study, it was shown that VEGF is critical for the initial s.c. growth of T-47D breast

carcinoma cells transplanted into nude mice, whereas other angiogenic factors such as bFGF can compensate for the loss of VEGF after the tumors have reached a certain size (Yoshiji, H., et al., Cancer Research 57:3924-28, 1997). VEGF is a major mediator of aberrant endothelial cells (EC) proliferation and vascular permeability in a variety of human pathologic situation, such as tumor angiogenesis, diabetic retinopathy and rheumatoid arthritis (Benjamin, L.E., et al., Proc Natl Acad Sci 94:8761-66, 1997, Soker, S., et al., Cell 92:735-745, 1998). VEGF induces expression of plasminogen activator (PA), PA inhibitor 1 (PAI-1), MMP, and interstitial collagenase in EC. These findings are consistent with the proangiogenic activities of VEGF. VEGF promotes expression of VCAM-1 and ICAM-1 in EC, thus may facilitate the adhesion of activated NK cells to EC. VEGF may promote monocyte chemotaxis (Pepper, M.S., et al., BBRC 181:902-906, 1991; Ferrara, N., et al., Endocr Rev 18:4-25, 1997). Tumors are believed to be the principal source of VEGF. A correlation has been observed between VEGF expression and vessel density in human breast tumors, renal cell carcinoma and colon cancer (Fong, T.A.T., et al., Cancer Res 59:99-106, 1999). VEGF and PGF expressions were significantly upregulated in 96% and 91% of hypervascular renal carcinoma tissues compared with adjacent normal kidney tissues (Takahashi, A., et al., Cancer Res 54:4233-7, 1994).

VEGF-B is a mitogen for EC and may be involved in angiogenesis in muscle and heart (Olofsson, B., et al., Proc Natl Acad Sci USA 93:2576-81, 1996). *In vitro*, binding of VEGF-B to its receptor VEGFR-1 leads to increased expression and activity of urokinase-type plasminogen activator and plasminogen activator inhibitor, suggesting a role for VEGF-B in the regulation of extracellular matrix degradation, cell adhesion, and migration (Olofsson, B., et al., Proc Natl Acad Sci USA 95:11709-14, 1998).

VEGF-C may regulate angiogenesis of lymphatic vasculature, as suggested by the pattern of VEGF-C expression in mouse embryos (Kukk, E., et al., Development 122:3829-37, 1996). Although VEGF-C is also a ligand for VEGFR-2, the functional significance of this potential interaction is unknown. Overexpression of VEGF-C in the skin of transgenic mice resulted in lymphatic, but not vascular, endothelial proliferation and vessel enlargement, suggesting the major function of VEGF-C is through VEGFR-3 rather than

VEGFR-2 (Jeltsch, M., et al., Science 276:1423-5, 1997). Using the CAM assay, VEGF and VEGF-C were shown to be specific angiogenic and lymphangiogenic growth factors, respectively (Oh, S.J., et al., Del Biol 188:96-109, 1997).

VEGF-D is a mitogen for EC. VEGF-D can also activate VEGFR-3. It is possible that VEGF-D could be involved in the regulation of growth and/or differentiation of lymphatic endothelium (Achen, M.G., et al., 1998 Proc Natl Acad Sci USA 95:548-53, 1998).

PIGF can potentiate the action of low concentrations of VEGF *in vitro* and *in vivo* (Park, J.E., et al., J Biol Chem 269:25646-54, 1994).

VEGFR-1 signaling pathway may regulate normal endothelial cell-cell or cell-matrix interactions during vascular development, as suggested by a knockout study (Fong, G.H., et al., Nature 376:65-69, 1995). Although VEGFR-1 has a higher affinity to VEGF than VEGFR-2, it does not transduce the mitogenic signals of VEGF in ECs (Soker, S., et al., Cell 92:735-745, 1998).

VEGFR-2 appears to be the major transducer of VEGF signals in EC that result in chemotaxis, mitogenicity and gross morphological changes in target cells (Soker, S., et al., Cell 92:735-745, 1998).

VEGFR-3 has an essential role in the development of the embryonic cardiovascular system before the emergence of lymphatic vessels, as shown by a knockout study (Dumont, D.J., et al., Science 282:946-949, 1998).

Neuropillin-1 is a receptor for VEGF165. It can enhance the binding of VEGF165 to VEGFR-2 and VEGF165 mediated chemotaxis (Soker, S., et al., Cell 92:735-745, 1998).

Gene regulation of some of these genes has been investigated and is discussed herein below.

In situ hybridization demonstrated VEGF mRNA was present in transplanted tumor cells but not in tumor blood vessels, indicating that immunohistochemical labeling of tumor vessels with VEGF antibodies reflects uptake of VEGF, not endogenous synthesis. VEGF protein staining was evident in adjacent preexisting venules and small veins as early as 5 hours after tumor transplant and plateaued at maximally intense levels in newly induced

tumor vessels by approximately 5 days. In contrast, vessels more than approximately 0.5 mm distant from tumors were not hyperpermeable and did not exhibit immunohistochemical staining for VEGF. Vessel staining disappeared within 24-48 h of tumor rejection. These studies indicate that VEGF is synthesized by tumor cells *in vivo* and accumulates in nearby blood vessels. Because leaky tumor vessels initiate a cascade of events, which include plasma extravasation and which lead ultimately to angiogenesis and tumor stroma formation, VEGF plays a pivotal role in promoting tumor growth (Dvorak, H.F., et al., J Exp Med 174:1275-8, 1991). In addition, it was shown that stromal cells can be stimulated by transplanted tumor cells for VEGF production (Fukumura, D., et al., Cell, 94:715-25, 1998). Fibroblasts cultured *in vitro* are highly activating for VEGF promoter function compared with fibroblasts in freshly isolated tumors, indicating the culture condition did not mimic the status of normal (unactivated) tissue *in vivo* (Fukumura, D., et al., Cell, 94:715-25, 1998). For example, C6 tumor spheroids (C6 is a cell line derived from a rat glial tumor -- C6 cells aggregate and form small spheroids in culture) implanted into nude mice became neovascularized accompanied by a gradual reduction of VEGF expression (Shweiki, D., et al., Proc Natl Acad Sci 92:768-772, 1995). The VEGF promoter region bears many of the characteristics of a house-keeping gene (Tischer, E., JBC 266:11947-11954, 1991), hence it is likely that almost any cell type could serve as a source for VEGF upon hypoxic or ischemic demand (Fukumura, D., et al., Cell, 94:715-25, 1998).

VEGF expression was upregulated by hypoxia (Shweiki, D., et al., Nature 359:843-5, 1992), due to both increased transcriptional activation and stability of its mRNA (Ikeda, E., et al., JBC 270:19761-5, 1995). In a number of *in vitro* studies, it was shown that hypoxia upregulates VEGF expression through the activation of PI3K/Akt pathway (Mazure, N.M., et al., Blood 90:3322-31, 1997) and HIF-1 (an enhancer induced by hypoxia and bind to VEGF promoter region) (Forsythe, J.A., MCB 16:4604-13, 1996; Mazure, N.M., et al., Blood 90:3322-31, 1997). VEGF is also upregulated by overexpression of v-Src oncogene (Mukhopadhyay, D., Cancer Res. 15:6161-5, 1995), c-SRC (Mukhopadhyay, D., et al., Nature 375:577-81, 1995), and mutant ras oncogene (Plate, K.H., Nature 359:845-8, 1992). The tumor suppressor p53 downregulates VEGF expression (Mukhopadhyay, D.,

Cancer Res. 15:6161-5, 1995). A number of cytokines and growth factors, including PGF, TPA (Grugel, S., et al., JBC 270:25915-9, 1995), EGF, TGF-b, IL-1, and IL-6 induce VEGF mRNA expression in certain type of cells (Ferrara, N., et al., Endocr Rev 18:4-25, 1997). Kaposi's sarcoma-associated herpesvirus (KSHV), which encodes a G-protein-coupled receptor -- a homolog of IL-8 receptor, can activate JNK/SAPK and p38MAPK and increase VEGF production, thus causing cell transformation and tumorigenicity. (Bais, C., Nature 391:86-9, 1998).

The growth of androgen-dependent Shionogi carcinoma in immunodeficient mice was regressed after the mice were castrated, accompanied by decrease in VEGF expression. Two weeks after castration, a second wave of angiogenesis and tumor growth begins with a concomitant increase in VEGF expression. (Jain, R.K., Proc Natl Acad Sci USA 95:10820-5, 1998).

VEGF-D is induced by transcription factor c-fos in mouse (Orlandini, M. Proc Natl Acad Sci 93:11675-80, 1996).

Overexpression of some of these genes has been evaluated using different systems.

VEGF overexpression in skin of transgenic mice induces angiogenesis, vascularhyperpermeability and accelerated tumor development (Larcher, F., et al., Oncogene 17:303-11, 1998). Retina tissue-specific VEGF overexpression in transgenic mice cause intraretinal and subretinal neovascularization (Okamoto, N., et al., Am J Pathol 151:281-91, 1997). VEGF overexpression mediated by the Tet system promotes tumorigenesis of C6 glioma cells when transplanted into nude mice. The tumors become hypervascularized with abnormally large vessels, arising from excessive fusions. The tumors were less necrotic. After VEGF expression was shut off, regression of the tumors occurred due to detachment of endothelial cells from the walls of preformed vessels and their subsequent apoptosis. Vascular collapse further lead to hemorrhages and extensive tumor necrosis (Benjamin, L.E., et al., Proc Natl Acad Sci 94:8761-66, 1997). In human-VEGF-promoter-GFP transgenic mice, implantation of solid tumor induces specific GFP expression in stromal cells. Transgenic mice were mated with T-antigen mice (able to form spontaneous mammary tumors) to generate double transgenic mice, in which spontaneous mammary tumors were

formed. Strong stromal, but not tumor, expression of GFP was observed (Fukumura, D., et al., Cell, 94:715-25, 1998). A CCD camera was used to monitor GFP expression. GFP half life was shown to be between about 1.2-1.5 days (Fukumura, D., et al., Cell, 94:715-25, 1998). The transgene was integrated into the IgG locus of the chromosome through DNA recombination (Fukumura, D., et al., Cell, 94:715-25, 1998). FVB derived VEGF-GFP transgenic mice were mated with wild-type C3H mice to create hybrid mice that can be served as hosts for C3H derived tumor lines (Fukumura, D., et al., Cell, 94:715-25, 1998).

VEGF-C overexpression in the skin of transgenic mice resulted in lymphatic, but not vascular, endothelial proliferation and vessel enlargement (Jeltsch, M., et al., Science 276:1423-5, 1997).

Neuropillin-1 overexpression in transgenic mice resulted in embryonic lethality. The embryos possessed excess capillaries and blood vessels. Dilated vessels and hemorrhage were also observed (Kitsukawa, T., et al., Development 121:4309-18, 1995).

The functions of some of these genes have been evaluated in knock-out mice constructs, animal studies, and *in vitro* studies.

A VEGF knockout was an embryonic lethal. F1 is also embryonic lethal and angiogenesis was impaired. VEGF secretion from +/- ES cells was reduced to 50% (Carmellet, P., et al., Nature 380:435-439, 1996; Ferrara, N., et al., Nature 380:439-442, 1996).

VEGFR-1 was evaluated in a lacZ knock-in wherein a fragment of the exon that contains ATG start codon was replaced by LacZ. Knockout mice were embryonic lethal. Blood vessels were formed, but the organization of the blood vessel was perturbed (Fong, G.H., et al., Nature 376:65-69, 1995).

VEGFR-2 was an embryonic lethal caused by defective endothelial cell development (Shalaby, F., et al., Nature 376:62-65, 1995).

VEGFR-3(LacZ Knock-in) was an embryonic lethal caused by defective blood vessel development (Dumont, D.J., et al., Science 282:946-949, 1998).

Neuropillin-1 was an embryonic lethal (Dumont, D.J., et al., Science 282:946-949, 1998).

In vitro studies showed that a mutant VEGF (a heterodimer of two mutant VEGF) (Siemeister, G., et al., Proc Natl Acad Sci 95:4625-9, 1998), as well as a GST-Exon7 (VEGF) fusion protein (Soker, S., et al., JBC 272:31582-88, 1997), was able to inhibit endothelial cell proliferation by acting as an VEGF antagonist and interfering VEGF binding to VEGFR-2 and VEGFR-1 (Siemeister, G., et al., Proc Natl Acad Sci 95:4625-9, 1998). More importantly, A VEGF neutralizing chimeric protein, containing the extracellular domain of VEGF receptor (either VEGFR-1 or VEGFR-2) fused with IgG, substantially reduced the development of retinal neovascularization when injected into mice with ischemic retinal disease (Aiello, L.P., et al., Proc Natl Acad Sci 92:10457-61, 1995).

Treatment of tumors with monoclonal antibodies directed against VEGF resulted in dramatic reduction in tumor mass due to the suppression of tumor angiogenesis (Kim, K.J., et al., Nature 362:841-44, 1993). Injection of antibodies against VEGF reduced tumor vascular permeability and vessel diameter in immunodeficient mice transplanted with human glioblastoma, colon adenocarcinoma, and melanoma (Yuan, F., et al., Proc Natl Acad Sci 93:14765-70, 1996). Retrovirus mediated overexpression of a dominant negative form of VEGFR-2 in nude mice suppresses the growth of transplanted rat C6 glioma tumor cells (Millauer, B., et al., Nature 367:576-9, 1994) mammary, ovarian tumors and lung carcinoma (Millauer, B., et al., Cancer Res 56:1615-20, 1996).

Mouse VEGFR-2 Promoter

The subject nucleic acids of the present invention (e.g., as described in Example 3) find a wide variety of applications including use as hybridization probes, PCR primers, expression constructs useful for compound screening, detecting the presence of VEGFR-2 genes or varients thereof, detecting the presence of gene transcripts, detecting or amplifying nucleic acids encoding additional VEGFR-2 promoter sequences or homologs thereof (as well as, structural analogs), and in a variety of screening assays.

The present invention provides efficient methods of identifying pharmacological agents or lead compounds for agents active at the level of VEGFR-2 gene transcription. A

wide variety of assays for transcriptional regulators can be used based on the teaching of the present specification, including, but not limited to, cell-based transcription assays, screening *in vivo* in transgenic animals, and promoter-protein binding assays. For example, the disclosed luciferase reporter constructs are used to transfect cells for cell-based transcription assays. For example, primary endothelial cells are plated onto microtiter plates and used to screen libraries of candidate agents for lead compounds which modulate the transcriptional regulation of the VEGFR-2 gene promoter, as monitored by luciferase expression (Example 5).

As noted above, the present invention relates to a recombinant nucleic acid molecule comprising the promoter region of a mouse VEGFR-2 gene. This invention provides a nucleic acid molecule having a sequence selected, for example, from the following groups: (a) a nucleic acid sequence of greater than 80% identity to that of SEQ ID NO:32, or a fragment thereof, exhibiting promoter activity, in particular VEGFR-2 promoter activity; (b) a nucleic acid sequence substantially complementary to said nucleic acid sequence of (a), or a fragment thereof; and (c) a nucleic acid sequence that specifically hybridizes to said nucleic acid sequences of (a) or (b) or fragments thereof.

The invention includes further VEGFR-2 promoter sequences identified based on the teachings of the present specification (including, but not limited to, sequence information and isolation methods, e.g., Example 3).

This invention also provides novel deletion constructs of the VEGFR-2 promoter which either increase or decrease promoter activity beyond that of the naturally occurring promoter. Such constructs may provide greater sensitivity than the native promoter when used to screen for compounds which affect VEGFR-2 promoter activity.

The nucleic acid molecules of this invention are useful in effecting tissue specific expression in endothelial cells, as well as, for screening for compounds that selectively modulate transcription in endothelial cells and compounds that modulate angiogenic processes.

Those skilled in the art can practice the invention by following the guidance of the specification supplemented with standard procedures of molecular biology for the isolation

and characterization of the VEGFR-2 promoters, their transfection into host cells, and vascular endothelial cell-specific expression of heterologous DNA operably linked to said VEGFR-2 promoters. For example, DNA is commonly transferred or introduced into recipient mammal cells by calcium phosphate-mediated gene transfer, electroporation, lipofection, viral infection, and the like. General methods and vectors for gene transfer and expression may be found, for example, in M. Kriegler, Gene Transfer and Expression: A Laboratory Manual, Stockton Press (1990). Direct gene transfer to cells *in vivo* can be achieved, for example, by the use of modified viral vectors, including, but not limited to, retroviruses, adenoviruses, adeno-associated viruses and herpes viruses, liposomes, and direct injection of DNA into certain cell types. In this manner, recombinant expression vectors and recombinant cells containing the novel VEGFR-2 promoters of the present invention operably linked to desired heterologous gene can be delivered to specific target cells *in vivo*. See, e.g., Wilson, Nature, 365: 691-692 (1993); Plautz et al, Annals NY Acad. Sci., 716: 144-153 (1994); Farhood et al, Annals NY Acad. Sci., 716: 23-34 (1994) and Hyde et al Nature, 362: 250-255 (1993). Furthermore, cells may be transformed *ex vivo* and introduced directly at localized sites by injection, e.g., intra-articular, intracutaneous, intramuscular and the like.

Cloning and characterization of the VEGFR-2 promoter are described in Examples 3 and 5, below.

Compound/Drug Screening

Another aspect of this invention is its use in screening for pharmacologically active agents (or compounds) that modulate VEGFR-2 receptor promoter activity either by affecting signal transduction pathways that necessarily precede transcription or by directly affecting transcription of the VEGFR-2 gene.

For screening purposes an appropriate host cell, preferably an endothelial cell, more preferably a vascular endothelial cell, is transformed with an expression vector comprising a reporter gene (e.g., luciferase) operably linked to the VEGFR-2 gene promoter of this invention. The transformed host cell is exposed to various test substances

and then analyzed for expression of the reporter gene. This expression can be compared to expression from cells that were not exposed to the test substance. A compound which increases the promoter activity of the VEGFR-2 promoter will result in increased reporter gene expression relative to the control. Similarly, compounds which act as antagonists for the VEGFR-2 promoter signalling pathway will result in decreased reporter gene expression relative to the control.

Thus, one aspect of the invention is to screen for test compounds that regulate the activity of the VEGFR-2 promoter by, for example, (i) contacting a host cell in which the VEGFR-2 promoter disclosed herein is operably linked to a reporter gene with a test medium containing the test compound under conditions which allow for expression of the reporter gene; (ii) measuring the expression of the reporter gene in the presence of the test medium; (iii) contacting the host cell with a control medium which does not contain the test compound but is otherwise essentially identical to the test medium in (i), under conditions essentially identical to those used in (i); (iv) measuring the expression of reporter gene in the presence of the control medium; and (v) relating the difference in expression between (ii) and (iv) to the ability of the test compound to affect the activity of the VEGFR-2 promoter.

Alternatively, the transformed cells may be induced with a transcriptional inducer, such as IL-1 or TNF-alpha, forskolin, dibutyryl-cAMP, or a phorbol-type tumor promoter, e.g., PMA. Transcriptional activity is measured in the presence or absence of a pharmacologic agent of known activity (e.g., a standard compound) or putative activity (e.g., a test compound). A change in the level of expression of the reporter gene in the presence of the test compound is compared to that effected by the standard compound. In this way, the ability of a test compound to affect VEGFR-2 transcription and the relative potencies of the test and standard compounds can be determined.

Thus in a further aspect, the present invention provides methods of measuring the ability of a test compound to modulate VEGFR-2 transcription by: (i) contacting a host cell in which the VEGFR-2 promoter, disclosed herein, is operably linked to a reporter gene with an inducer of VEGFR-2 promoter activity under conditions which allow for expression of the reporter gene; (ii) measuring the expression of the reporter gene in the absence of the test

compound; (iii) exposing the host cells to the test compound either prior to, simultaneously with, or after contacting, the host cells with the inducer; (iv) measuring the expression of the reporter gene in the presence of the test
compound; and (iv) relating the difference in expression between (ii) and (iv) to the ability of the test compound to modulate VEGFR-2-mediated transcription.

Because different inducers are known to affect different modes of signal transduction, it is possible to identify, with greater specificity, compounds that affect a particular signal transduction pathway. Further, because the VEGF receptors have been shown to be upregulated in tumor cells and this upregulation appears to be necessary for tumor angiogenesis, such assays provide a means of identifying compounds that will inhibit and/or reverse tumor growth by downregulating VEGFR-2 expression and thus preventing or reducing tumor angiogenesis.

A variety of reporter genes may be used in the practice of the present invention. Preferred are those that produce a protein product which is easily measured in a routine assay. Suitable reporter genes include, but are not limited to chloramphenicol acetyl transferase (CAT), light generating proteins (e.g., luciferase), and beta-galactosidase. Convenient assays include, but are not limited to calorimetric, fluorimetric and enzymatic assays. In one aspect, reporter genes may be employed that are expressed within the cell and whose extracellular products are directly measured in the intracellular medium, or in an extract of the intracellular medium of a cultured cell line. This provides advantages over using a reporter gene whose product is secreted, since the rate and efficiency of the secretion introduces additional variables which may complicate interpretation of the assay. In a preferred embodiment, the reporter gene is a light generating protein. When using the light generating reporter proteins described herein, expression can be evaluated accurately and non-invasively as described above (see, for example, Contag, P. R., et al., (1998) Nature Med. 4:245-7; Contag, C. H., et al., (1997) Photochem Photobiol. 66:523-31; Contag, C. H., et al., (1995) Mol Microbiol. 18:593-603).

In another aspect of this invention, transgenic animals expressing a heterologous gene encoding a detectable product under the regulatory control of

the VEGFR-2 promoter, as disclosed herein, may be used to determine the effect of a test compound on the stimulation or inhibition of the VEGFR-2 promoter *in vivo*. The test compound is, for example, administered to the animal and the degree of expression of the heterologous gene observed is compared to the degree of expression in the absence of administration of the test compound using, for example, whole animal luciferase-based assays as disclosed herein. Methods of generating transgenic animals were described above.

This invention also provides transgenic animals useful as disease models for studying VEGFR-2 function and endothelial cell-specific gene expression.

Various forms of the different embodiments of the invention, described herein, may be combined.

The following examples are intended only to illustrate the present invention and should in no way be construed as limiting the subject invention.

EXAMPLES

Example 1
Generating the Targeting Cassette and Vector

A. Creation of the Backbone Vector

pTK53: The 0.5 kb mouse phosphoglycerate kinase 1 promoter was amplified with PGK primers (PGKF, SEQ ID NO:1: ATCGAATTCTACCGGGTAGGGGAGGCGCTTT; PGKR, SEQ ID NO:2: GGCTGCAGGTCGAAAGGCCCGGAGATGAGG) using mouse genomic DNA (Genome Systems, Inc., St. Louis, MO) as template. This fragment was then double digested with EcoRI and PstI and cloned into the pKS vector (Stratagene, La Jolla, California) which was linearized with the same enzymes. The neomycin gene was amplified with NeoF (SEQ ID NO:3: ACCTGCAGCCAATATGGGATCGGCCATTGAAC) and NeoR (SEQ ID NO:4: GGATCCGCGGCCGCCCCCAGCTGGTTCTTTCCGCCTC) primers using pNTKV1907 (Stratagene) as a template. The 1.1 kb PCR fragment was double

digested with PstI and BamHI and cloned into the pKS-PGK vector which was linearized with the same enzymes. This pKS-PGK-Neo vector was used to clone thymidine kinase gene as follows. Primers TKF (SEQ ID NO: 5: GGATCCTCTAGAGTCGAGCAGTGTGGTTTT) and TKR (SEQ ID NO:6: GAGCTCCCGTAGTCAGGTTTAGTTCGTCCG) were used to amplify the TK gene from pNTKV1907 (Stratagene). The amplified 2kb fragment was then digested with BamHI and SacI and cloned into pKS-PGK-Neo vector that was linearized with the same enzymes. This constructed vector was designated as pTK. A synthetic linker F5R5 was made after annealing of two primers (forward primer, SEQ ID NO: 7: GTACATTTAAATCCTGCAGG, reverse primers, SEQ ID NO:8: AGCTCCTGCAGGATTTAAAT). This linker was inserted between Asp718I and HindIII sites of pTK and the new construct was designated pTK5. A second synthetic linker F3R3 was made by annealing of two primers (F3R31 forward primer, SEQ ID NO:9: GGCCCGGGCTTAATTAATGCATCATATGGTACCGTTTAAACGCGGCCGCAAGCT TGTCGACGGCGCGCCGGCCGGCC, F3R32reverse primer, SEQ ID NO:10: GATCGGCCGGCCGGCGCGCCGTCGACAAGCTTGCGGCCGCGTTTAAACGGTACC ATATGATGCATTAATTAAGCCCG).). This linker was inserted between NotI and BamHI sites of pTK and the new construct was designated pTK53. Schematics of the vectors are shown are shown in Figure 1.

B. Introduction of Luciferase

pTK-LucYG and pTK-LucR: The yellow green luciferase gene was isolated from pGL3 vector (Promega) as a HindIII-SalI fragment and was cloned into pGK53 that was linearized with the same enzymes. The new construct was designated pTK-LucYG (8931 bp), shown in Figure 2.

The red luciferase gene was isolated from pGL3-red vector (Dr. Christopher Contag, Stanford University, Stanford, Calif.) as a HindIII-SalI fragment and was cloned into pGK53 that was linearized with the same enzymes. The new construct was designated pTK-LucR (8931 bp), shown in Figure 2.

Example 2

Insertion of Targeting Sequences

A. Generation of vitronectin targeting vector: The targeting construct pTKLR-Vn was generated by inserting vitronectin (VN) DNA sequences into pTK-LucR vector.

Vitronectin (VN) is an abundant glycoprotein present in plasma and the extracellular matrix of most tissues. In a previous study, it was shown that heterozygous mice carrying one normal and one null VN allele and homozygous null mice completely deficient in vitronectin demonstrate normal development, fertility, and survival. This suggests that VN is not essential for cell adhesion and migration during normal mouse development (Zheng, X., et al., Proc Natl Acad Sci U S A 1995 92:12426-30). Mouse vitronectin genomic DNA sequence of 5004 bp was obtained from GenBank database (Accession number X72091). Based on this sequence, a 1.63 kb 3'end vitronectin fragment was amplified (reverse primer VN1R, SEQ ID NO:11: CTGTATTTAAATCTGCCCACCCTATTCAGGACAGTAGTC; forward primer VN1F, SEQ ID NO:12: CCAATGCATCAACCCAGCCAGGAGGAGTGCG) using mouse C57BL/6 genomic DNA as template (Genome Systems, Inc., St. Louis, MO). This fragment was digested with *SwaI* and *NsiI* and cloned into pTK-LucR (linearized with *SwaI* and *SbfI*). This construct was designated as pTK-LucR3. Subsequently, a 2.35 kb 5'end vitronectin fragment was amplified (reverse primer VN2R, SEQ ID NO:13: AACGCGTCGACTTCGGAGATGTTTCGGGGATAACCAGG, forward primer VN2F, SEQ ID NO:14: TTGGCGCGCCCCATAGAGAAGAGACACCAAAGGCACGCTC) using mouse C57BL/6 genomic DNA as template. This fragment was digested with *SalI* and *AscI* and cloned into pTK-LucR vector that was linearized with *SalI* and *AscI*. This construct was designated as pTKLR-Vn. Figure 3A shows the restriction map of pTKLR-Vn vector. The polylinker between the neomycin gene and red luciferase gene is used to insert the VEGF promoter or other promoters of interests. The predicted homologous recombination between pTKLR-Vn and vitronectin gene is illustrated in Figure 3B. Upon insertion of the VEGF-

LucR transgene cassette, the endogenous vitronectin gene is destroyed. Figure 3C shows the genomic DNA sequence of VN.

B. Generation of Fos targeting vector: The targeting construct pTKLG-Fos was generated by inserting FosB DNA sequences into pTK-LucYG vector.

FosB is one of the members of the Fos family. It plays a functional role in transcriptional regulation. It has been shown that FosB mice are born at a normal frequency, are fertile and present no obvious phenotypic or histologic abnormalities (Gruda et al (1996) *Oncogene* 12:2177-2185). A 28.8 kb genomic region that contains mouse FosB DNA sequence was obtained from GenBank database (Accession number AF093624).

Using this sequence, a 1.71 kb 5'end FosB fragment was amplified (forward primer FosB1F, SEQ ID NO:15: CTGTATTTAAATCCCGTTTCTCACTGTGCCTGTGTC; reverse primer FosB1R, SEQ ID NO:16: GTCTCCTGCAGGCTTCCTCCTCCTTGTTCCTTGCG) using mouse C57BL/6 genomic DNA as template. This fragment was digested with *SwaI* and *SbfI* and cloned into pTK-LucYG vector that was linearized with *SwaI* and *SbfI*. This construct was designated as pTK-LucYG3. Subsequently, a 1.58 kb 3'end FosB fragment was amplified (forward primer FosB2F, SEQ ID NO:17: AACGCGTCGACGGATGGGATTGACCCCCAGCCCTC; reverse primerFosB2R, SEQ ID NO:18: TTGGCGCGCCCCTTGCCTCCACCTCTCAAATGC) using mouse C57BL/6 genomic DNA as template. This fragment was digested with *SalI* and *AscI* and cloned into pTK-LucYG vector that was linearized with *SalI* and *AscI*. This construct was designated as pTKLG-Fos (Figure 4A). The polylinker between the neomycin gene and red luciferase gene is used to insert the VEGFR2 promoter (Example 3, Figures 5A-C, enhancer Figure 11), Tie2 promoter (Example 3, Figure 15, enhancer Figure 16), as well as, other promoters of interests. The predicted homologous recombination between the targeting vector bearing the VEGFR2 promoter (Figure 14) or the Tie2 promoter (Figure 19) and FosB gene is also illustrated. As shown in the Figures, the VEGFR2-LucYG transgene cassette and Tie2-LucYG transgene cassette is inserted downstream of FosB gene translational stop signal.

Therefore, the targeted transgenic mice should still have a functional FosB gene while expressing the transgenes. Figure 4B showns the DNA sequence of FosB.

Example 3

Insertion of Promoter Sequences of Interest

A. pTKLR-Vn/VEGF: Mouse VEGF genomic DNA sequence of 2240 bp that contains a partial VEGF promoter region was obtained from GenBank (accession number: U41383). Accordingly, primers were designed to amplify a 0.69 kb (VF1-VR1A; Table 1) and a 0.98 kb fragment (VF2-VR2; Table 1). It was confirmed that each pair of primers can amplify the predicted product using mouse129SvJ genomic DNA as template.

Table 1

Name	SEQ ID NO:	Sequence
VF1	19	ACCTC ACTCT CCTGT CTCCC CTGAT TCCCA A
VR1A	20	GCTCT GGCGG TCACC CCCAA AAGCA
VF2	21	CCCTT TCCAA GACCC GTGCC ATTTG AGC
VR2	22	ACTTT GCCCC TGTCC CTCTC TCTGT TCGC
KF1	23	GCTGC GTCCA GATTT GCTCT CAGAT GCG
KR1	24	TTCTC AGGCA CAGAC TCCTT CTCCG TCCCT
KF2	25	CAGAT GGACG AGAAA ACAGT AGAGG CGTTG GC
KR2	26	GAGGA CTCAG GGCAG AAAGA GAGCG
TF3	27	AGCTT AGCCT GCAAG GGTGG TCCTC ATCG
TF2	28	CAAAT GCACC CCAGA GAACA GCTTA GCCTG C
TR1	29	GCTTT CAACA ACTCA CAACT TTGCG ACTTC CCG

Conditions for PCR amplification are shown in Figure 6. These primers were used for PCR screening of mouse 129/SvJ genomic DNA BAC (bacterial artificial chromosome) library (Genome Systems, Inc., St. Louis, MO). The library, on average, contained inserts of 120 kb with sizes ranging between 50 kb to 240 kb. A large genomic DNA fragment that

contained VEGF promoter region was obtained. Southern blot analysis was performed to map the VEGF promoter region. A unique HindIII restriction site was mapped approximately 7.8 kb upstream of the ATG translational start codon of the VEGF gene. The sequences between HindIII and ATG translational start codon are inserted into the polylinker of pTKLR-Vn vector to finish the construction of targeting vector that contains VEGF-LucR transgene (Figure 3A).

B. VEGFR2 targeting vector pTKLG-Fos-KPN

1. Cloning of VEGFR2 promoter

Mouse VEGFR2 genomic DNA sequence of 1079 bp that contains partial VEGFR2 promoter region was published previously (Ronicke et al (1996) *Cir. Res.* 79:277-285). Accordingly, primers that were able to amplify a 0.45 kb (KFI-KR1; Table 1) and a 0.58 kb fragment (KF2-KR2; Table 1) were designed. It was confirmed that each pair of primers can amplify the predicted product using mouse 129SvJ genomic DNA as template. DNA sequences for these primers are shown in Table 1 above. PCR amplification conditions are shown in Figure 6. These primers were used for PCR screening of mouse 129/SvJ genomic DNA BAC library. A large genomic DNA fragment of VEGFR2 promoter region was obtained. Based on the VEGFR2 restriction map that was published (Ronicke et al, *supra*), a 4.6 kb HindIII-Xbal fragment that covers the VEGFR2 promoter region was subcloned from the VEGFR2 BAC clone into the pSK vector (Stratagene, La Jolla, CA) and linearized with Hindlll and Xbal. This construct was designated pSK-K6.

2. Engineering of the VEGFR2 promoter

PSK-K6 was engineered to deleted a 159 bp sequence of the 3'end promoter region spaning from ATG translational start codon to an Xbal site. A 0.3 kb 3'end fragment was amplified by PCR (Forward primer (VR2F): CGCTAGTGTGTAGCCGGCGCTCTC (SEQ ID NO:30); reverse primer (VR2R): ATAAGAATGCGGCCGCCTGCACCTCGCGCTGGGCACAG (SEQ ID NO:31)) and digested with Bsu361 and Notl. This fragment was used to replace the 0.45 kb Bsu36I-Notl

fragment of pSK-K6 and the resulting construct was designated PSK-KP, which contains VEGFR2 promter sequences of 4.5 kb, spanning from a HindIII site to the ATG translational start codon. The 4.5 kb VEGFR2 promoter was fully sequenced and the sequence is shown in Figures 5A-C (SEQ ID NO:32). The present invention includes, but is not limited to, an isolated polynucleotide having at least 90%, preferably 92%, more preferably 95%, and even more preferably 98% sequence identity to the sequence presented as SEQ ID NO:32.

3. Cloning of VEGFR2 enhancer

In a recent report, it was described that a 5 1 1 bp sequence within the first intron of VEGFR2 gene functions as an endothelial cell specific enhancer. (Kappel et al (1999) *Blood* 12: 4284-4292). Accordingly, this VEGFR2 enhancer sequence was amplified by PCR using VEGFR2 BAC clone DNA. (Forward primer (VEF): ACACGCCTCGAGAAATGTGCTGTCTTTAGAAGCCACTG (SEQ ID NO:33); Reverse primer (VER): ACACGCGTCGACGATCCAATAGGAAAGCCCTTCCATAAAC (SEQ ID NO:34)).This fragment was digested with Xhol and Sall and cloned into the Sall site of the pSK vector. The resulting construct was designated PSK-KN. The 511 bp VEGFR2 enhancer is shown in Figure 11 (SEQ ID NO:35).).

4. pGL3B2

The yellow-green luciferase containing vector pGL3B (Promega, Madison, WI) was re-engineered as illustrated in Figure 12. First, pGL3B was digested with NotI and then blunt ended with T4 DNA polymerase. A PmeI linker (New England Biolab) was then ligated into the vector. The new vector, pGL3B-Pme, was double digested with Asp718 and HindIII and ligated with a synthetic linker resulted from annealing of two complementary oligos. (GL3B-Forward GTACTTAATTAAGCTTGGTACCCGGGGCGGCCGC (SEQ ID NO:36); GL3B-Reverse AGCTGCGGCCGCCCCGGGTACCAAGCTTAATTAA (SEQ ID NO:37)). The new vector was designated pGL3B2.

Construction of pGL3B2-KPN

As illustrated in Figure 12, the VEGFR2 promoter is isolated from pSK-KP as a HindIII-NotI fragment and cloned into the pGL3B2 vector that is linearized with HindIII and NotI. The new construct was designated pGL3B2-KP. Subsequently, the VEGFR2 enhancer was isolated from pSK-KN as a XhoI-SalI fragment and cloned into the pGL3B2-KP vector that was linearized with SalI. The new construct was designated pGL3B2-KPN.

6. Construction of pTKLG-Fos-KPN

The VEGFR2 promoter-luciferase-enhancer cassette was isolated from pGL3B2-KPN as a PacI-SalI fragment and cloned into the pTKLG-Fos vector that was linearized with Paci and SalI. The new construct was designated pTKLG-Fos-KPN. (Fig 13), Using this targeting construct, the VEGFR2 promoter-Luciferase-enhancer transgene cassette is targeted to the FosB gene locus through homologous DNA recombination, as illustrated in Fig 14.

C. Tie2 targeting vector pTKLG-Fos-TPN

1. Cloning of Tie2 promoter

A 477 bp region of the mouse Tie2 promoter has been isolated and sequenced. (Fadel et al (1998) *Biochem J.* 330:335-343). Using this region, primers that were able to amplify a 0.45 kb (TF3-TR1; Table 1) and a 0.47 kb fragment (TF2-TR1; Table 1) were designed. It was confirmed that each pair of primers amplified the predicted product using mouse 129SvJ genomic DNA as template. DNA sequences for these primers are shown in Table 1 above and PCR amplification conditions are shown in Fig 6. These primers were used for PCR screening of mouse 129/SvJ genomic DNA BAC library. A large genomic DNA fragment of Tie2 promoter region was obtained. Based on the Tie2 genomic DNA restriction map that was published (Dumont et al (1994) *Genes and Development* 8:1897-1909), a 10.5 kb Asp718-EcoRV fragment spanning the Tie2 promoter region was subcloned from the Tie2

BAC clone into the pSK vector linearlized with Asp718 and EcoRV. The new construct was designated pSK-T67.

2. Engineering of the Tie2 promoter

pSK-T67 was further engineered to delete all the 3.4 kb sequence spaning from ATG translational start codon to EcoRV site. A 1.0 kb 3'end promoter region was amplified by PCR (T2 Forward primer: TATCAACACTCGGGAGGCTGAGGGAG (SEQ ID NO:38); T2 reverse primer: ATAAGAATGCGGCCGCACTTCCCCAGATCTCCCCATCCAGC (SEQ ID NO:39)) and digested with BstAPI and NotI. The 0.55 kb BstAPI-NotI fragment was used to replace the 4.0 kb BstAPI-NotI fragment of pSK-T67 and the resulting construct was designated PSK-TP, which contains Tie2 promter sequences of 7.1 kb, spanning from a Asp718 site to the ATG translational start codon. The 7.1 kb Tie2 promoter was fully sequenced and the sequence was shown in Figure 15 (SEQ ID NO:40).). The present invention includes, but is not limited to, an isolated polynucleotide having at least 90%, preferably 92%, more preferably 95%, and even more preferably 98% sequence identity to the sequence presented as SEQ ID NO:40.

3. Cloning of Tie2 enhancer

In a previous report, it was described that a 1.7 kb region within the first intron of the Tie2 gene functions as an endothelial cell specific enhancer. (Schiaeger et al (1997) *PNAS USA* 94: 3058-3063). Accordingly, this 1.7 kb Tie2 enhancer region was subcloned from the Tie2 BAC clone DNA as a XhoI-Asp718 fragment into the pSK vector that was linearized with the same enzymes. The Asp718 site was then converted to a SalI site using a SalI linker (New England Biolab). The resulting construct was designated PSK-TN. The 1.7 kb Tie2 enhancer was fully sequenced and the sequence is shown in Figure 16 (SEQ ID NO:41).).

4. Construction of pGL3B2-TPN

As illustrated in Figure 17, the Tie2 promoter was isolated from PSK-TP as a Asp718-NotI fragment and cloned into the pGL3B2 vector that was linearized with Asp718 and NotI. The new construct was designated pGL3B2-TP. Subsequently, the Tie2 enhancer was isolated from PSK-TN as a XhoI-SalI fragment and cloned into the pGL3B2-TP vector linearized with SalI. The new construct was designated pGL3B2-TPN.

5. Construction of pTKLG-Fos-KPN

The Tie2 promoter-Luciferase-enhancer cassette is isolated from pGL3B2-TPN as a PacI-SalI fragment and cloned into the pTKLG-Fos vector linearized with PacI and SalI. The new construct was designated pTKLG-Fos-TPN. (Figure 18). Using this targeting construct, the Tie2 promoter-Luciferase-enhancer transgene cassette is targeted to the FosB gene locus through homologous DNA recombination, as illustrated in Fig 19.

Example 4

Generation of Transgenic Mice Carrying the Constructs of the Present Invention

A. General Procedure: Figure 7 depicts a generalized description of generation of transgenic mice using the targeted transgenic vectors described in Example 3. Details regarding embryonic stem (ES) cell culture, transfection, blastocyst injection and implantation to a pseudopregnant foster are described, for example, in Hogan et al (1994) "Manipulating the Mouse Embryo, A Laboratory Manual. Second Edition", Cold Spring Harbour Laboratory Press.

After construction the targeted transgenic construct are transfected into C57BL/6 embryonic stem (ES) cells. (Genome System Inc., Genome Systems, Inc., St. Louis, MO) through electroporation. The antibiotic G418 is used to select for cells in which the DNA construct containing the Neo gene is integrated, either randomly or by homologous recombination. The nucleoside analog gancyclovir is converted by TK to a cytotoxic derivative. DNA that has integrated by homologous recombination lose the TK gene and are

resistant to the drug, whereas cells that have incorporated the DNA randomly are likely to retain the TK gene. Thus, cells containing random integrations into a chromosomal location that allows the expression of the TK gene are killed. The G418 and gancyclovir resistant clones are then be screened by PCR and Southern blot analysis and those that have homologous DNA recombination is used for FVB/N blastocyst injection (Genome System, Inc.). Between 4-16 blastocysts are transferred to the uterus of a pseudopregnant foster mother. The pups are typically born 17 days after the transfer. Either random bred mice or F1 hybrid mice make suitable recipients. Females of certain random-bred stocks (*e.g.*, CD1 mice, from Charles River Laboratories) have very large ampullae, which makes oviduct transfer easier. These mice also generally make good mothers. Alternatively, F1 hybrid females (*e.g.*, B6 x CBA F1) can be used as recipients. Although their ampullae are smaller, make exceptionally good mothers,rearing litters as small as two pups. See, for example, Hogan et al. (1994), *supra.*

B. Screening for homologous DNA recombination positive ES cells

1). pTKLG-Fos/VEGFR2: Analysis of homologous DNA recombination between pTKLG-Fos/VEGFR2 targeting vector and the FosB gene is carried out using Southern blot analysis as shown in Figure 8. Genomic DNA prepared from G418 resistant ES cells is digested with PvuII and probed with probe A to confirm the 5'end DNA recombination. PvuII digestion of DNA bearing homologous recombination reveals two separate bands of 8.2 and 4.0 kb, whereas digestion of DNA from homologous recombination negative clones reveals only the 8.2 kb band. The 3'end of DNA recombination is tested by hybridizing NotI digested DNA with probe B. NotI digestion of DNA bearing homologous recombination will reveal two separate bands of >8.2kb and 5.0 kb, whereas digestion of DNA from homologous recombination negative clones will only reveal the >8.2 kb band. Once homologous DNA recombination is confirmed, positive clones is selected for FVB/N blastocyst injection.

2) pTKLG-Fos/Tie2: Analysis of homologous DNA recombination between pTKLG-Fos/Tie2 targeting vector and the FosB gene is analyzed by Southern blot in a similar manner as described above for pTKLG-Fos/VEGFR2. Once homologous DNA recombination is confirmed, positive clones are selected for FVB/N blastocyst injection.

3) PTKLR-Vn/VEGF: Analysis of homologous DNA recombination between pTKLR-Vn/VEGF targeting vector and the vitronectin gene is analyzed by PCR. DNA primers designed according to the predicted homologous recombination, are listed in Table 2.

Table 2
PCR primers for analysis of homologous DNA recombination between pTKLR-Vn/VEGF targeting vector and the vitronectin gene

5'end primers
F51 5'- CCCAGTGTCTCTGATTTAGGGAGAGCACCTGAG -3' (SEQ ID NO:42)
R51 5'- CCAGACTGCCTTGGGAAAAGCGCCTC -3' (SEQ ID NO:43)
F52 5'- CAGTGAGAGTCTTCTCTGTCCCTCAATCGGTTCTG -3' (SEQ ID NO:44)
R52 5'- TGGATGTGGAATGTGTGCGAGGCCAG -3' (SEQ ID NO:45)
3'end primers
F31 5'- AATCAAAGAGGCGAACTGTGTGTGAGAGGTCC -3' (SEQ ID NO:46)
R31 5'- CGGCTCCCCAAAATGTGGAAGCAAGC -3' (SEQ ID NO:47)
F32 5'- GAATCCATCTTGCTCCAACACCCCAACATC -3' (SEQ ID NO:48)
R32 5'- CGCCTCCTCTCCCCAGTCTCCCCTTG -3' (SEQ ID NO:49)

Primers F51-R51 and F52-R52 amplify a 1799 bp and a 1841 bp DNA fragment respectively from the 5'end of the transgene that is integrated into the vitronectin site through homologous DNA recombination, whereas primers F31-R31 and F32-R32 amplify a 3549 bp and a 3428 bp DNA fragment respectively from the 3'end of the transgene that is integrated into the vitronectin site through homologous DNA recombination. Clones that

allow successful amplification of both the 5'end and 3'end of the integrated transgene are selected for FVB/N blastocyst injection.

C. Analysis of chimeric mice

The pups developed from injected blastocysts contain chimeras, as can be identified by their agouti coat color when an ES cell derived from a mouse having a dark coat color (e.g., C57BL/6) is injected into the blastocyst of a light coat color animal (e.g., FVB/N, genotype B/B). DNA analysis (*e.g.,* Southern blotting, PCR) is conducted to further confirm the presence of the transgene in these pups as described above in Section B. These animals may be obtained commerically, for example from The Jackson Laboratory, Bar Harbor, MN.

D. Generating targeted transgenic C57BL/6 mice with white coat color

Breeding of the chimeric mice generates homozygous targeted transgenic mice, as depicted in Figure 9. The targeted mice are used to monitor gene expression through the measurement of luciferase mediated light emission from the mice. In a preferred embodiment, the targeted mouse has a light coat color (e.g., white coat color), because the black colored coat (an example of a dark coat color) of C57BL/6 mice can absorb light emitted from the body and may interfere the sensitivity of the bioluminescence assay. An inbred mouse strain C57BL/6-Tyr C2j/+ strain (Jackson Laboratory, Bar Harbor, MN) is available for this purpose. This strain of mice have white color coat, yet they still have the same genetic background as C57BL/6 mice except that the gene responsible for the black coat color is mutated. Unfortunately, C57BL/6-Tyr C2j/+ ES cells are not currently available. Therefore, the designed breeding program illustrated in Figure 9 is aimed to generate mice that are homozygous for the target transgene and have white coat color. C57BL/6 ES cells are prepared as described above and introduced into a suitable blastocyst (*e.g.,* from the FVB/N strain of mice). The blastocysts are implanted into a foster mother. Chimeric mice are shown in Figure 9 as white animals with black and green patches. Chimeric animals are bred with C57BL/6-Tyr C2j/+ mice to create F1 hybrids. Subsequent breeding of the F1 hybrids generates several type of mice, including the one that is

homozygous for the target transgene and has a white coat color (shown in Figure 9 as b/b; L/L), which is used for *in vivo* gene regulation monitoring.

A C57BL/6 mouse and a C57BL/6-Tyr C2j/+ mouse are considered to be substantially isogenic. Accordingly, the method of the present invention exemplified in Figure 9 provides a means for generating breeding groups of substantially isogenic mice in a selected genetic background carrying at least one transgene of interest.

E. Dual luciferase targeted transgenic mice

As described above, two targeting vectors are generated. PTKLR-Vn carries a red luciferase gene and is targeted into vitronectin locus. PTKLG-Fos carries a yellow-green luciferase gene and is targeted into FosB locus. A number of promoters, including VEGF promoter, VEGFR2 promoter, and Tie2 promoter are cloned into these vectors, as described above. Subsequently three type of targeted transgenic mice are generated. VEGF mice carry VEGF promoter-red luciferase transgene (VEGF-LucR) integrated into vitronectin locus. VEGFR2 mice carry VEGFR2 promoter-yellow-green luciferase (VEGFR2-LucYG) transgene integrated into FosB locus. Tie2 mice carry Tie2 promoter-yellow-green luciferase (Tie2-LucYG) transgene integrated into FosB locus. Through a breeding program illustrated in Figure 10, dual luciferase targeted transgenic mice are produced, carrying both of the VEGF-LucR and the VEGFR2-LucYG transgenes. The degradation of luciferin by yellow-green luciferase and red luciferase generates lights that emit at 540 nM and 610 nM respectively. These wavelengths of light are measured individually using a photo-counting camera (intensified CCD). Therefore, both VEGF expression and VEGFR2 expression, for example, can then be monitored in the same mouse at the same time.

Example 5

Modulation of Expression Mediated by VEGFR2 Promoter Sequences

The 4.5 kb VEGFR2 promoter identified in Example 3 and shown in Figures 5A-C (SEQ ID NO:32) was cloned into the polylinker of pGL3B2 (Fig. 12) to control the transcription of luciferase coding sequences (Fig. 12). A 0.5 kb VEGFR2 enhancer

sequence was cloned down stream of the luciferase to enhance endothelial specific expression. The resulting expression construct (pGL3B2-KPN; Fig. 12) was used to transiently transfect primary bovine endothelial cells (Clonetics) using lipofectamine (Promega). The cells were seeded onto 12-well plastic culturing plates (Nunc) prior to transfection. The transfection was carried out according to the manufacture's instructions (Promega). Plasmid pRL-TK (Promega), containing *Renilla* luciferase driven by the thymidine kinase promoter, was used as an internal control in all transfection experiments. The primary bovine endothelial cells were cultured in EGM-2 MV medium (Clonetics) at 37^0C in 5% CO_2, 95% air. After transfection, the cells were lysed with passive lysis buffer (Promega) and assayed with the Dual-Luciferase Reporter Assay System (Promega) for luciferase activity.

Several angiogenesis and neoplasticity inhibitors (Sigma) were tested for their effects on the expression of VEGFR2 expression in primary bovine endothelial cells transiently transfected with pGL3B2-KPN as described above. Briefly, 24 hrs after transfection, the cells were treated with selected angiogenesis and neoplasticity inhibitors for 36 hrs and assayed for luciferase activity. The tested compounds included the neoplasticity inhibitor Mithramycin, and angiogenesis inhibitors 2-Methoxyestradiol, Thalidomide, and Fumagillin. At least some of the tested compounds had the effect of reducing luciferase expression mediated by the 4.5 kb VEGFR2 promoter.

These results suggest that sequences derived from the 4.5 kb VEGFR2 promoter are useful for screening for compounds capable of modulating VEGFR2-mediated angiogenesis.

Example 6

Modulation of Expression Mediated by Tie2 Promoter Sequences

The 7.1 kb Tie2 promoter identified in Example 3 and shown in Figure 15 (SEQ ID NO:40) was cloned into the polylinker of pGL3B2 (Fig. 17) to control the transcription of luciferase coding sequences (Fig. 17). The resulting expression construct (pGL3B2-TP; Fig. 17) was used to transiently transfect primary bovine endothelial cells (Clonetics) using lipofectamine (Promega). The cells were seeded onto 12-well plastic culturing plates

(Nunc) prior to transfection. The transfection was carried out according to the manufacture's instructions (Promega). Plasmid pRL-TK (Promega), containing *Renilla* luciferase driven by the thymidine kinase promoter, was used as an internal control in all transfection experiments. The primary bovine endothelial cells were cultured in EGM-2 MV medium (Clonetics) at 37^0C in 5% CO_2, 95% air. After transfection, the cells were lysed with passive lysis buffer (Promega) and assayed with the Dual-Luciferase Reporter Assay System (Promega) for luciferase activity.

Several angiogenesis and neoplasticity inhibitors (Sigma) were tested for their effects on the expression of Tie2 expression in primary bovine endothelial cells transiently transfected with pGL3B2-TP as described above. Briefly, 24 hrs after transfection, the cells were treated with selected angiogenesis and neoplasticity inhibitors for 36 hrs and assayed for luciferase activity. The tested compounds included the neoplasticity inhibitor Mithramycin, and angiogenesis inhibitors 2-Methoxyestradiol, Thalidomide, and Fumagillin. At least some of the tested compounds had the effect of reducing luciferase expression mediated by the 7.1 kb Tie2 promoter.

These results suggest that sequences derived from the 7.1 kb Tie2 promoter are useful for screening for compounds capable of modulating Tie2-mediated angiogenesis.

As is apparent to one of skill in the art, various modification and variations of the above embodiments can be made without departing from the spirit and scope of this invention. These modifications and variations are within the scope of this invention.